

REVIEWS

The Challenge of Drying Method Selection for Protein Pharmaceuticals: Product Quality Implications

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ABSTRACT: Numerous drying methods are used to dry solutions of proteins in the laboratory and/or in pharmaceutical manufacturing. In this review article, we will discuss many of these drying methods. We will briefly introduce and compare the unit operations involved in the drying methods to give an insight on thermal history, and the different stresses that a drying method can present to an active ingredient, particularly for protein molecules. We will review and compare some important physico-chemical properties of the dried powder that result from using different drying methods such as specific surface area, molecular dynamics, secondary structure (for protein molecules), and composition heterogeneity. We will discuss some factors that might lead to differences in the physico-chemical properties of different powders of the same formulation prepared by different techniques. We will examine through a literature review how differences in some of these properties can affect storage stability. Also, we will review process modifications of the basic drying methods and how these modifications might impact physico-chemical properties, in-process stability and/or storage stability of the dried powders. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 96:1886–1916, 2007

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INTRODUCTION

Whether processing conditions during drying are altered or different drying methods are used to prepare the same formulation, the net result is modification of the thermal history. In polymer science, the effects of thermal history and drying method have been shown to influence relaxation

time, diffusivity, stability, and glass transition temperature.¹ In the food industry, different drying methods commonly used in the pharmaceutical industry have been shown to affect product quality. For example, spray drying and freeze drying produced ovalbumin powders with different emulsification and foaming properties upon reconstitution.² Freeze drying caused greater losses in volatile ingredients in *Petrosselinum crispum* than traditional oven drying and 'shade' drying, even at ambient temperatures.³ Vacuum oven drying produced dried pectin powder of much lower solubility over a pH range of 2–10 as compared to freeze drying and spray drying.⁴ Other issues such as color loss,⁵ flow

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properties,⁶ and preservation of labile ingredients such as vitamin C^{5,7} and β -carotene⁷ have also been a function of the drying method.

Spray drying and freeze drying are by far the two most popular methods of drying protein solutions in the pharmaceutical industry. Supercritical fluid technology, spray freeze drying (SFD), spray coating, and modifications of spray coating such as drying with conventional spouted bed,⁸ and other drying methods based on solvent evaporation without atomization (such as vacuum drying, Xerovac[®], foam drying, film drying) are also used but on a much smaller (mostly research) scale.^{9–15} A few studies have been published in the pharmaceutical field that compare the effects of different drying methods on product properties. We will review some of this published work. It is important to investigate the effect of drying method and processing conditions on pharmaceutical properties of amorphous formulations. One can expect differences in the physico-chemical properties among powders of the same formulation dried by different methods, and even differences between their storage stability profiles have been documented.^{16–18} In the pharmaceutical industry, drying is employed if the storage stability of the active ingredient (physical and/or chemical) in solution is unsatisfactory. The choice of drying method will then be associated mainly with the economics of drying and the intended route of administration. For example, spray-dried powders are commonly prepared when the intended route of administration is via inhalation, since the desired control over particle size can be obtained, but freeze drying is commonly employed for injectable products since sterility and “particle-free” quality attributes are more easily obtained in freeze drying. Improving storage stability is often focused on re-formulation, which in many times can be tedious and time consuming, but variations in processing specifics can also address stability issues. However, a full understanding and appreciation of the stresses encountered

during drying, and secondly the influence of the drying method (or thermal history) on the physico-chemical properties of the dried formulations is needed to choose the appropriate drying process and to optimize this process for maximum product quality.

UNIT OPERATIONS, PROCESS VARIABLES, AND THERMAL HISTORIES INVOLVED IN DIFFERENT DRYING METHODS

Most drying methods involve removal of solvent by sublimation, evaporation or a combination of both. In this review, we classify the drying methods according to the main mechanism of solvent removal.

Drying by Evaporation

Spray Drying

Spray drying is a one-step economic drying method widely used to produce powders for pulmonary delivery.^{19–21} The unit operations involved in spray drying are summarized in Figure 1. Briefly, the drying method in a spray dryer involves feeding the solution through an atomizer nozzle placed inside the drying chamber at a controlled rate. As the liquid stream emerges from the nozzle orifice, large liquid–air interfacial expansion will occur and the stream breaks up into small fine droplets (atomization) in the drying chamber by the aid of an atomizing gas (air or inert gas such as nitrogen). Viscosity, surface tension, and density of the liquid, as well as atomizing gas flow rate and pressure, influence the break up of the liquid and hence droplet size distribution.²² Depending on the design of the atomizer nozzle, the liquid stream will mix with the atomizing gas right before or right after it emerges out of the nozzle orifice into the drying chamber. Other methods of atomization that have

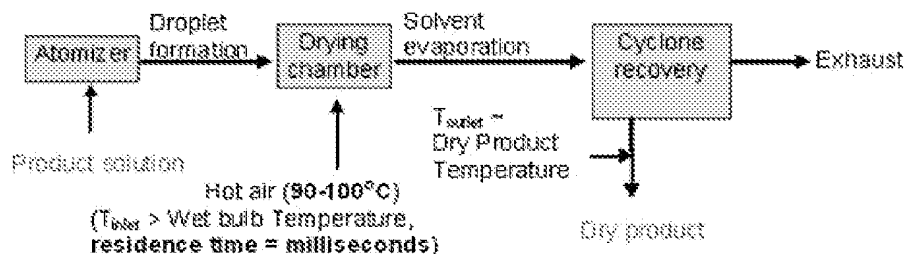


Figure 1. Unit operations involved in spray drying.

been used involve the application of high ultrasonic frequencies. Different designs of atomizer nozzles allow for control of droplet size and droplet size distribution. Upon contact with hot dry air or hot inert dry gas such as nitrogen inside the drying chamber, evaporation occurs from the atomized droplets and the resulting dried particles are collected after passing through a cyclone separator into a collecting tube. In a typical spray dryer, drying gas is not re-circulated but exhausted to the atmosphere (open cycle). On the other hand, drying gas is re-circulated in a 'closed cycle' system. Variables that can be controlled during the process include inlet temperature (T_{inlet}), liquid feed rate, atomizing air flow rate, drying air flow rate, and relative humidity of the inlet air. Outlet temperature (T_{outlet}) depends on all four previous parameters.^{23,24} Both product temperature and temperature inside the drying chamber correspond more closely to T_{outlet} , even 5 cm below the nozzle.²⁴ Excellent reviews on spray drying and the types of atomizers used can be found in literature.^{19–26}

Drying of a spray droplet has been divided into two phases.^{24,27} A schematic for the temperature–time profile of both the droplet surface and droplet interior is presented in Figure 2. In the first phase (constant rate period), the rate of

evaporation is constant and the liquid feed temperature increases rapidly to the wet bulb temperature (T_{wb}).²⁷ T_{wb} is the lowest temperature attained by the surface of the droplets during evaporation and is dependent upon the evaporative cooling effect. The surface of the droplets would maintain 100% relative humidity in a system at T_{wb} .²⁸ As the relative humidity in the inlet air stream is decreased, drying rate increases, evaporative cooling increases and T_{wb} decreases. The temperature of the interior of the droplet is usually 10–15°C lower than T_{wb} .²⁹ Dissolved solute is transported via diffusion and convection to the droplet surface. This phase lasts until a solid phase (crust) begins to form at the surface of the droplet, usually within 10^{-4} to 10^{-3} s.^{24,27} At this point, solute concentration at the surface has reached a critical solute concentration (C_{crit}) that depends on the solubility of the solute at T_{wb} (S_{wb}).²⁷ At C_{crit} , diffusion of the solvent through this barrier is slower than the heat transfer, and the system enters the second phase of drying. Drying and evaporation rate decline in the second phase (falling rate phase) and particle size does not undergo further reduction.^{24,27} The temperature of the particle will rise until it equals the dry bulb temperature of air (T_{db}), concurrently the temperature of the particle interior will rise to T_{db} . T_{db} is the temperature of

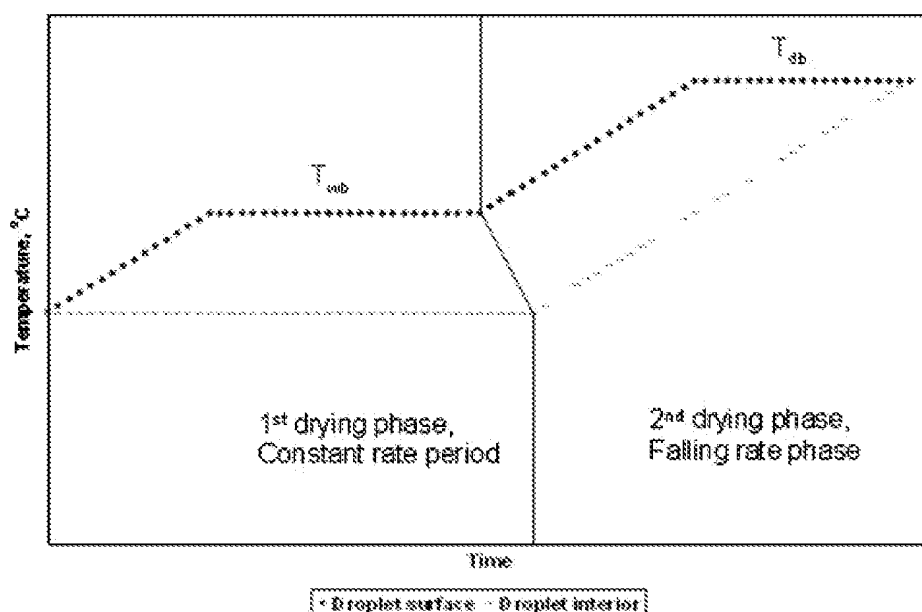


Figure 2. A schematic for the drying phases involved during spray drying. Temperature profile as a function of time for both the droplet surface and droplet interior (adopted from Hsu et al.²⁸).

the surface of the dried particles after evaporation of most of the water, and is usually close to T_{outlet} . The particle remains at the drying-air outlet-temperature before collection.

Spray Coating

This drying method has been used on a small scale to produce powders of diphtheria toxoid and alum-adsorbed hepatitis-B surface antigen for needle-free injection,³⁰ as well as for drying recombinant human deoxyribonuclease (rhDNase) formulations^{28,31} and subtilisin.³² Drying in a spray coater involves first fluidization of an inert carrier (such as lactose or hydroxypropylmethyl cellulose seed particles) of defined particle size range in the drying chamber. This is followed by atomization of the liquid feed into the chamber to deposit on the carrier. While atomizing, the coated carrier particles are concurrently dried by hot air, leaving a film residue of the dried formulation containing the active ingredient and collected in a powder collector. The powder can be sieved later and particles can be separated based on particle size range. Specific surface area in this drying process is therefore limited by particle size of the seed particles.

During spray coating and evaporation, the outer surface of the coating droplet might have a temperature profile similar to that of the droplet during spray drying. On the other hand, the inside of the coating droplet in contact with the seed particles (unlike the interior of the sprayed droplets during spray drying) is exposed to the high temperature of the seed particles fluidized in the chamber prior to atomization. The outcome

is more residence time for the interior of the coated droplet with hot seed particles before evaporation is complete, relative to droplets in a spray drying process, and therefore a greater probability of thermal denaturation for heat sensitive materials.^{28,33}

Drying by Evaporation without Atomization

In cases where the active ingredient has been known to be vulnerable to the atomization step prior to drying (due to creation of a large air-water interface), alternative drying methods without atomization were developed. These methods are based on evaporation from a solution under reduced pressure such as vacuum drying,^{11,12} foam drying,^{13–15} and Xerovac.³⁴ Film drying, a form of controlled evaporative drying, was addressed by Franks et al.¹⁰ and has been referred to as liquid film dehydration by Shroeder and Schwarz.⁹ The equipment necessary to perform evaporative drying consists of a drying chamber, a condenser, and a vacuum pump (i.e., a conventional freeze dryer may be used).^{13–15} The principle unit operations are summarized in Figure 3. Solutions are simply dried at ambient temperatures under reduced pressure. Solutions will dry into a foam or a film, depending on the initial solids content before drying and the pressure.^{13–15} If the starting solute concentration is high enough ($\sim 20\%$ w/v or more), solutions will foam initially when the “primary” drying cycle is initiated (i.e., foaming during a reduction of the chamber pressure from atmospheric conditions). The “primary drying” step normally involves evaporation of most of the bulk water $\sim 90\%$ or

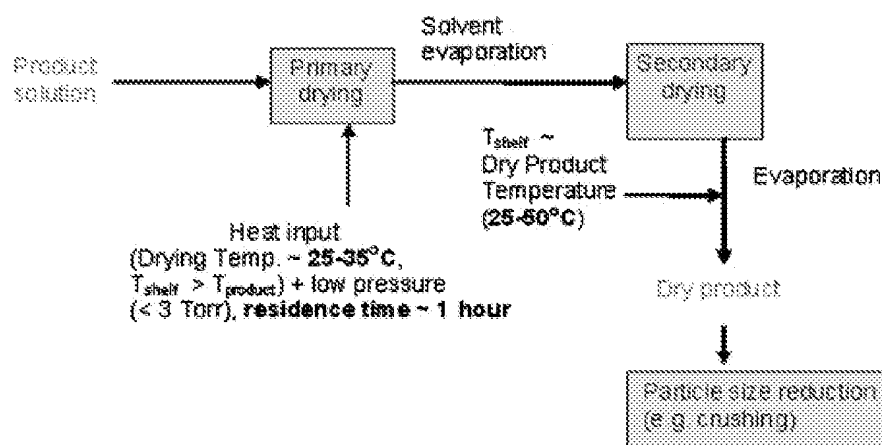


Figure 3. Unit operations for drying methods based on evaporation without prior solution atomization.

more during the first hour.³⁵ Mass flux for water vapor is expected to be very high during “primary drying.” A “secondary drying” step must be followed to further reduce the moisture content, which might require a long drying time (up to a few days) due to the very low specific surface area of the “film” or “foam,” unless an additional particle size reduction step is included (such as crushing, jet milling, etc.).

Drying by Sublimation

Freeze Drying (Lyophilization)

Freeze drying unit operations are covered in great depth in the literature.^{36–42} Freeze drying is unique among drying methods in that after freezing, the material is dried as a solid, that is, below T'_g , which is preferable from a stability point of view. On the other hand, it is a longer process as compared to, for example, spray drying and spray coating. Although less economic compared to other drying methods, freeze drying is the most common drying method for producing powders for parenteral administration. Freeze drying has also been used to produce fast dissolving tablets.⁴³

The unit operations involved in freeze drying are summarized in Figure 4. Freeze drying consists of three main stages: freezing, primary drying, and secondary drying. A schematic for the temperature–time profile in a typical freeze drying run during the different stages is presented in Figure 5. Freezing is a short step that lasts for a few hours, during which most of the desiccation has occurred, producing a mixture of ice and solid product commonly containing roughly 20% water and all solutes in a single amorphous phase. In the primary drying step, solvent is removed via sublimation of ice. This step can require anywhere from a few hours up to several days, depending on the glass transition temperature of the freeze

concentrate (T'_g) and solute concentration. Primary drying should always be performed 2–5°C below the collapse temperature (T_c) (or below T'_g) to avoid product collapse and maintain elegant cake structure. Too high a ‘safety margin’ ($\leq 1^\circ\text{C}$ below T'_g) could result in collapse in a subgroup of product vials that are subjected to more radiative heat transfer than other vials (e.g., edge vials). Too low a ‘safety margin’ ($\geq 5^\circ\text{C}$ below T'_g) would needlessly prolong primary drying, and hence freeze drying cycle, time. After primary drying has ended and all ice has been removed, a secondary drying step follows whose purpose is to remove the remaining water in the amorphous phase ($\sim 20\text{--}30\%$ w/w water)^{42,44} via desorption at much higher product temperatures, normally above ambient. Heating from primary drying to secondary drying final temperature should be slow (and in many cases can be done in several steps) to avoid a situation in which the shelf temperature is higher than the glass transition temperature (T_g) of the product thereby leading to collapse (melting) of the product (since T_g increases very slowly relative to the shelf temperature depending on the rate of evaporation of water from the amorphous phase). Secondary drying is usually completed in shorter times than required for primary drying.

In freeze drying, mass transfer of water molecules within the dried layer is accomplished via bulk flow caused by a pressure gradient and by diffusion.⁴⁵ Heat transfer is accomplished mainly through gas conduction (vapor in the space between the shelf and the bottom of the vial). Conduction from shelf to vial at points of contact and radiation (from top, bottom, and sides to the vial) also are significant mechanisms for heat transfer.⁴⁶

An additional “annealing” step can be included during freezing. Annealing is a process by which frozen samples are held at a temperature between the ice melt temperature and T'_g for a period of time. Annealing is usually done to crystallize out

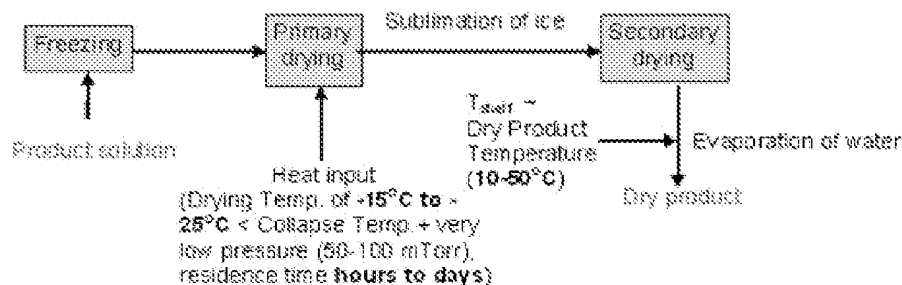


Figure 4. Unit operations involved in freeze drying.

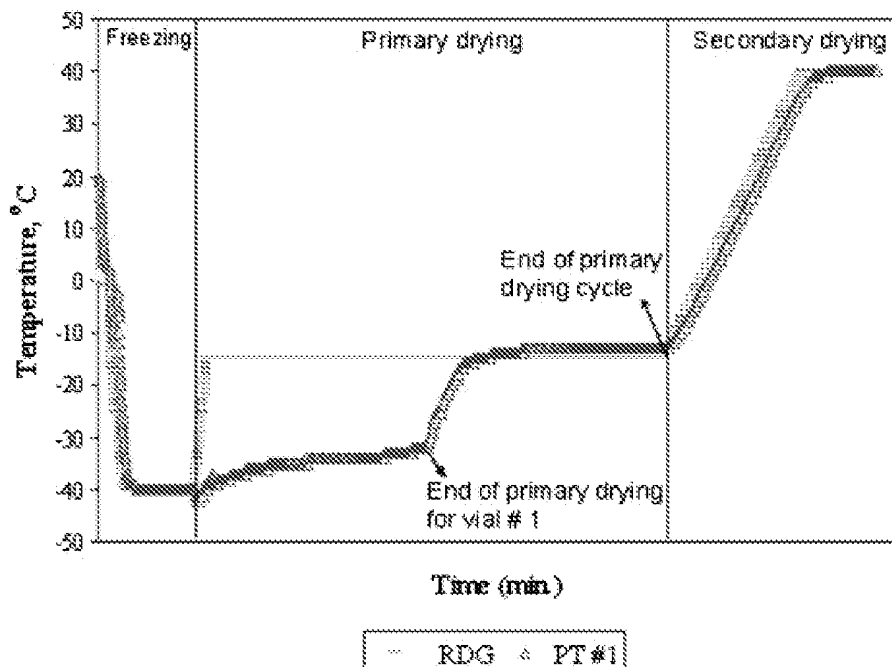


Figure 5. A schematic for the drying phases involved during freeze drying. Temperature profile as a function of time for both the shelf (RDG, dash) and one vial of the product (PT #1, hollow triangles). Note how the end of primary drying for the particular vial monitored, observed by the rise of PT #1 up to the shelf temperature, is before the operational end of the primary drying.

bulking agents such as mannitol (hold at $\sim -23^{\circ}\text{C}$ for several hours)⁴⁷ or glycine,⁴⁸ as well as to increase the size of ice crystals, and therefore increase the size of pores through which water vapor can flow, thereby decreasing primary drying times.

Spray Freeze Drying

SFD is a recent method developed as an alternate drying method to spray drying for precise control of particle size in powders for pulmonary delivery with improved aerodynamic properties for enhanced delivery to the deep lung. Examples of proteins that have been successfully prepared as pulmonary dry powder formulations using SFD are rhDNase and anti-IgE monoclonal antibody (anti-IgE MAb).²⁰ SFD was also used to produce dried powders with a higher density than those produced by spray drying for epidermal powder immunization with vaccines.⁴⁹ SFD shares common unit operations and drying stresses with both freeze drying and spray drying. The first step involves atomization of an aqueous solution of the active ingredient with additives into a cold vapor phase over a cryogenic liquid (commonly liquid

nitrogen) to form droplets. Droplets may begin to freeze in the cold vapor phase before contact with the cryogenic liquid, but completely freeze upon contact with the cryogenic liquid. One modification for freezing the droplets has been atomization of the liquid feed directly into the cryogenic liquid rather than over the liquid, thereby eliminating the effect of air–water interface on protein stability. The rate of freezing of the droplets in either case is rapid. To our knowledge, there has been no attempt to control the rate of freezing in SFD. Frozen particles are collected and subjected to a freeze drying cycle as described before with or without annealing above T'_g prior to primary drying.

Drying by Precipitation

Supercritical Fluid (SFL) Technology

SFL drying has recently been introduced to the pharmaceutical field and represents a drying method in which solvent removal is accomplished neither by sublimation nor by evaporation. SFL technology is described in great detail in an excellent review by Jovanovic et al.⁵⁰ As the name

implies, the basis of SFL is using supercritical fluids (SCF), largely because the precipitation may be controlled by control of pressure. A SCF is formed when a gas such as CO₂ is liquefied above a certain critical temperature and pressure. The SCF is considered as an 'antisolvent' for numerous proteins in aqueous and organic solutions, and has been used to precipitate insulin, lysozyme, myoglobin, albumin, catalase, and alkaline phosphatase. Drying by SFL technology can be accomplished with or without an atomization step prior to drying. An atomization step will allow greater control of mass transfer rate, particle morphology, particle size, and specific surface area. The main concept is that a SCF acts as an extraction medium for water, thereby increasing solute concentration in solution. As a result, the solute (of protein and additives) will precipitate. Particles formed are dried by further extraction of the remaining solvent by the SCF. There are two reported methods for atomization in SCF drying: one is atomizing the protein solution into a vessel containing the SCF (supercritical fluid anti-solvent technique—SAS), and the other is atomizing the SCF together with the protein solution (effervescent atomization) into a vessel containing the SCF (rapid expansion of supercritical solutions—RESS).^{50–52} Mass transfer can be improved by increasing SCF to solvent ratio or decreasing droplet size.

MAIN CHALLENGES AND STRESSES DURING DRYING

The same stress encountered during one drying method may be also encountered in another drying method. For example, heating is a stress common to spray drying and spray coating, atomization (which results in the creation of a large air–water interface) and shearing stresses are common to spray drying and SFD, freezing stresses are common to both freeze drying and SFD, and stresses arising from dehydration are common to all drying processes whether sublimation or evaporation is involved in solvent removal.

High Temperature

Heat-induced degradation of thermolabile drugs is a risk associated with the use of high temperature during drying, particularly with biological materials such as proteins and vaccines. Spray drying and spray coating utilize heat from a hot

gas stream to evaporate solvent (either in the form of droplets as in spray drying or film of solvent coating seed particles in spray coating) created by the atomization of a continuous liquid feed. Most proteins undergo irreversible denaturation, and hence losses in biological activity and solubility, when exposed to high temperatures (thermal denaturation). When a protein solution is gradually heated above a critical temperature, it undergoes a sharp transition from the native state to the denatured state. The temperature at the transition midpoint, where the concentration ratio of native and denatured states is unity, is known either as the melting temperature (T_m) or the denaturation temperature (T_d). Depending on the protein structure and the severity of the heating, these changes may or may not be reversible. Water greatly facilitates thermal denaturation of proteins. Dry protein powders are extremely stable to thermal denaturation. The value of T_d and enthalpy of denaturation (ΔH_{hyd}) decrease rapidly as the water content is increased and some proteins are stable at temperatures well above 100°C if the moisture content is very low (<1% w/w),⁵³ for example, human growth hormone (hGH) and bovine growth hormone (bGH).^{54,55}

Thermal denaturation is not generally observed in spray drying because although the inlet air temperature may be high, due to the self-cooling effect caused by the solvent evaporation, the temperature of the dried product does not rise above T_{wb} . In addition, the temperature of the droplet interior is always lower than T_{wb} ,²⁹ development of high concentration increases viscosity and slows unfolding,⁵⁶ and perhaps most important, T_m increases dramatically as water content decreases. The extent of denaturation depends on temperature–time combinations that proteins encounter during the drying process, and the key point to avoid thermal denaturation is to avoid long contact times between droplets and hot air in the spray-dried chamber.⁵⁷ A 40 s exposure of tissue plasminogen activator (t-PA) solution to 50°C or a 20 s exposure to 80°C caused negligible degradation. A 40 s exposure to 80°C, however, caused 16% degradation.⁵⁸ Examples of proteins that have showed increased aggregation with an increase in T_{inlet} are β -galactosidase,^{33,59} oxyhemoglobin,⁶⁰ insulin,⁶¹ lactate dehydrogenase (LDH),⁶² and recombinant human growth hormone (r-hGH).⁵⁸ It is therefore a good practice to use a low T_{inlet} to reduce the potential of thermal stress to the protein. The primary

structure affects thermal stability of proteins, and proteins that contain a greater proportion of hydrophobic amino acid residues (especially valine, isoleucine, leucine, and phenylalanine) tend to be more stable than the more hydrophilic proteins. Other structural factors that contribute to relative stability of proteins against thermal denaturation are disulfide bonds and the presence of salt bridges buried in hydrophobic clefts.

While a few studies have explored the use of spray coating of proteins and vaccines in the pharmaceutical industry,^{28,30,31} it was found that rhDNase after spray coating showed a significant loss of bioactivity (65% bioactive) and high protein aggregation (10% soluble aggregates).^{28,31} The same solutions subjected to spray drying with the same T_{inlet} and atomizing air flow rate showed greater recovery and no aggregation post drying. Loss of bioactivity and aggregation were not associated with the air–water interface since rhDNase was stable to atomization. It seems likely that such differences arose from thermal denaturation during spray coating.

Freezing

Freezing step is the first step in freeze drying and SFD. Most of the desiccation is achieved during the freezing stage.^{39,63} Upon freezing a solution, a sequence of events occur: super-cooling, ice nucleus formation, and ice crystal growth.⁶⁴ Super-cooling is the retention of liquid state below the equilibrium freezing point of the solution.³⁹ Solidification occurs abruptly during super-cooling and latent heat is released.⁶⁵ The degree of super-cooling is the temperature difference between the equilibrium ice formation temperature and actual temperature at which ice begins to form,⁶³ and aqueous solutions can super-cool by 10°C or more.^{39,63} If equilibrium solubility of a solute has been exceeded at a low temperature, the solute may crystallize even if ice crystals have not formed, such as has been observed with pentamidine isethionate.⁶⁶ As temperature is lowered further after ice crystal formation, solutes become more concentrated in the regions between ice crystals and a critical temperature is reached below which solutes cannot crystallize on the process timescale, and the system will increase in viscosity sufficiently to transform into a solid amorphous system or glass containing roughly 20% water⁴² or a lyotropic liquid crystal phase may form.⁶⁷

Freezing creates many destabilizing stresses, especially for biological materials. The ‘dehydration effect’ after ice formation and removal of bulk water from the protein phase is one stress that reduces the hydrophobic interactions.⁶³ The formation of sharp ice crystals that can pierce intact structures of membrane viruses presents another potential stress.^{68,69} Variation in freezing rate can present different stresses to protein formulations, depending on the nature of the protein. Slow freezing has been shown to increase protein damage in systems prone to phase separation, since phase separation is often kinetically controlled.⁷⁰ High freezing rate (i.e., high super-cooling, e.g., by quenching in liquid nitrogen or dry-ice acetone bath) has traditionally been combined with very low temperatures, more super-cooling and the generation of smaller and greater number of ice crystals and thereby larger ice SSA.⁶³ Strambini and Gabellieri⁷¹ proposed that freezing-induced damage to proteins at the ice surface involves adsorption to the ice surface followed by partial unfolding. A relatively large ice–liquid interface correlated directly with high loss of secondary and tertiary structure during freezing. This phenomenon was found to be reversible, although a small fraction of the proteins studied did not recover enzymatic activity. Eckhardt et al.⁷² showed a relationship between aggregate formation and freezing rate (over a range of 0.5–50°C/min) after freeze-thaw cycles in several hGH formulations. Hsu et al. showed a relationship between freezing rate and the degree of turbidity of t-PA solutions upon reconstitution.⁷³ Chang et al.⁷⁴ reported increased level aggregation of phosphofructokinase, LDH, glutamate dehydrogenase, tumor necrosis factor, and interleukin-1 (IL-1) receptor antagonist when solutions of these proteins were quench frozen as opposed to slow cooling. Other proteins reported to be vulnerable to fast cooling rates are bovine IgG,⁷⁵ catalase,⁷⁶ and β -galactosidase.^{76,77} The addition of nonionic surfactants^{74,75} provided a high degree of protection against surface denaturation during freeze thawing of several proteins, thereby making surfactants effective as cryoprotectants. Of course in addition to the fact that surfactant molecules may be more surface active than protein molecules,⁷⁴ a reduction in the SSA of ice brought about by the addition of nonionic surfactants⁷⁸ may contribute to the protection of proteins from the damaging effect of the ice. Also, increasing protein concentration was found to largely counteract the damage caused by a large ice SSA. Hansson⁷⁹

reported a reduction in aggregate formation of human IgG during freeze-thaw upon increasing IgG concentration to 50 mg/mL. Similar results were reported for IL-1 receptor antagonist,⁷⁴ β -galactosidase,^{76,77} and LDH.⁷⁶

Freeze concentration presents another stress during the freezing stage since a highly concentrated solution presents an environment in which ionic strength increases to very high values and large pH shifts via selective buffer crystallization are facilitated. Protein-protein interactions increase due to high concentration, possibly leading to aggregation. A high ionic strength can promote degradation of proteins. For example, chemical degradation and physical aggregation of hGH, a predominantly α -helical protein, was found to be sensitive to the level of phosphate buffer in both solution⁸⁰ and in the solid state.⁸¹ Aggregation in the solid state was found to be maximum at a buffer level of 0.227 mg buffer/mg hGH whereas chemical degradation was found to be maximum at 0.114 mg buffer/mg hGH.⁸¹ The preceding results may arise from high ionic strength. Additionally, it was shown that NaCl was detrimental to stability, but this effect appeared to be more specific than just "ionic strength".⁸¹ A high ionic strength has also been demonstrated to cause osmotic dehydration of microorganisms.³⁹ Freeze concentration can accelerate the rates of second-order chemical reactions (i.e., during freezing) such that degradation may be faster degradation in a freeze concentrate in spite of the lower temperature; an example is the oxidation of ascorbic acid.^{37,82} A large pH shift can occur when one buffer component crystallizes out. pH shifts are affected by solution composition, solute concentration, and history of freezing.^{39,76} Such shifts can obviously be detrimental to proteins sensitive to pH shifts. The sodium phosphate buffer system shows a dramatic decrease in pH of about 4 pH units due to the crystallization of $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$.^{37,83-85} Other buffers such as citrate or potassium phosphate produce buffer systems show much smaller pH changes upon freezing.⁸³⁻⁸⁵ Therefore, selection of buffer type along with minimizing the buffer concentration, or eliminating the buffer entirely if the drug is self-buffering is advisable.^{36,63}

Under certain conditions,⁸⁶⁻⁸⁸ low temperatures have resulted in the cold denaturation of some proteins and enzymes such as β -lactoglobulin, phosphofructokinase, and pyruvate kinase.⁸⁹ Cold denaturation occurs due to the weakening of hydrophobic interactions with a decrease in

temperature and leads to the spontaneous unfolding of a protein. The conformation changes occurring at low temperatures may be partial or total destruction of tertiary structure while secondary structure remains intact.^{89,90} Although cold denaturation can be reversible, it does occur with irreversible loss of biological activity for some proteins.²⁶ Even some viral vaccines such as respiratory syncytial virus (RSV), influenza, measles, and rubella were unstable when exposed to very low temperatures after freezing.⁹¹⁻⁹³ The degree of damage was temperature and time dependent.⁹¹⁻⁹³

Annealing in the Frozen State

Annealing in the frozen state can be done during a freeze drying or SFD cycle after solutions have first been frozen. Annealing is done by holding at a temperature between the ice melt temperature and the glass transition temperature of the freeze concentrate, T'_g , for a period of time. Annealing allows larger ice crystals to grow at the expense of small ones in a process called Ostwald ripening.⁴⁷ Below T'_g , the system is in a glassy state and ice crystal growth is limited. Above T'_g , growth is possible. Annealing may also result in the crystallization of bulking agents such as glycine and mannitol, which leads to an increase in T'_g . As a result of annealing a purely amorphous system, primary drying cycles are shorter, a more homogeneous freeze-dried batch is obtained and the SSA of the freeze-dried product is significantly lower than the same formulation with no annealing.^{46,94-96}

The effects of annealing on protein secondary structure in the literature are variable. For example, Randolph et al.^{44,97} showed that annealing accelerated phase separation (and/or crystallization of one or more components) in the freeze-concentrated liquid phase thereby causing conformational changes in hemoglobin. On the other hand, annealing prior to primary drying caused a reduction in SSA and a more nativelike protein secondary structure in lyophilized formulations of recombinant human interferon- γ (rhIFN- γ)¹⁶ and bovine IgG.⁷⁵ Assessing conformational changes in protein structure by infrared spectroscopy will be discussed later in this review.

Atomization and the Air-Water Interface

Proteins, like many surfactants, are amphiphilic polyelectrolytes and therefore surface active, can

adsorb to surfaces and interfaces such as liquid–air and liquid–solid interfaces. In general, the driving force for protein adsorption is the increase in entropy of the water molecules upon adsorption. Water molecules are ordered around the hydrophobic protein domains when the protein molecules are in the bulk of the solution, and removal of the hydrophobic domains from bulk water removes this order. Proteins adsorb onto the air–water interface in several steps.^{98–100} The first step, known as the ‘induction period’ or ‘lag phase,’ involves diffusion of protein molecules from bulk solution to the subsurface region. During the ‘lag phase,’ surface tension (γ) of the solution remains nearly equal to that of the pure solvent. Induction times are not always observed, especially for highly concentrated solutions or rapidly adsorbing proteins.¹⁰⁰ Induction times depend on the nature of the protein. For example, Tripp et al.⁹⁹ reported that no induction time (at $C_B = 0.01$ mg/mL) was observed for hGH and bovine serum albumin (BSA). At the same C_B , myoglobin induction time was measured to be 13 min and that of ribonuclease A (RNase) was measured to be 300 min.

After the ‘lag phase,’ protein molecules transfer from the subsurface region to the air–water interface and adsorb. A rapid decrease in γ is observed during this phase. The latter phase will be the first regime observed if the ‘lag phase’ is absent, as was observed for hGH and BSA. γ will decrease until a ‘mesoequilibrium surface tension’ (MST) phase is reached where γ will remain relatively constant or may very slightly decrease due to conformational rearrangement of the adsorbed protein molecules. Attainment of steady state MST indicates that the adsorbed protein molecules have achieved their equilibrium conformation and surface concentration at the air–water interface. MST value depends on both nature of the protein and on the bulk concentration of the protein C_B .^{98,99} MST for hGH was measured to be 37.8 dynes/cm² at $C_B = 1$ mg/mL, 43.8 dynes/cm² at $C_B = 0.1$ mg/mL, and 47.7 dynes/cm² at $C_B = 0.01$ mg/mL. For BSA, MST was 52.7 dynes/cm² at $C_B = 1$ mg/mL, 53.8 dynes/cm² at $C_B = 0.1$ mg/mL, and 55.2 dynes/cm² at $C_B = 0.01$ mg/mL.⁹⁹ Adsorbed protein molecules at the surface and subsurface may be exchanged with bulk protein molecules (i.e., desorption of surface and subsurface molecules) and refold back in bulk solution.¹⁰¹ Alternately, surface adsorption can be irreversible due to slow refolding kinetics thereby leading to protein aggregation and denaturation.⁴¹

Proteins usually assume conformations in solution that bury nonpolar amino acids in the protein “core” to maintain minimum contact with water (the hydrophobic interaction), while more hydrophilic amino acids are exposed to the aqueous environment. If a protein is exposed to a hydrophobic interface (i.e., high interfacial tension) such as the air–water by means of shaking or atomization, or a hydrophobic liquid–water interface, or even to ice–liquid interfaces, it will orient its hydrophobic amino acids away from the aqueous environment.¹⁰² If allowed to remain at the interface for sufficient time or if there is rapid and large expansion of the air–liquid interface (such as with atomization), proteins (especially globular proteins) will unfold and expose as many hydrophobic groups as possible to the nonaqueous “side.” If refolding kinetics is slow, aggregation may follow by the interaction of hydrophobic groups of unfolded protein molecules together if refolding kinetics is slow.^{41,103} Aggregates may form through covalent and/or noncovalent interactions between protein molecules. Covalent bonds may be formed by esterification of carboxyl groups in aspartic acid and glutamic acid side chains with hydroxyl groups of serine or threonine residues. Carbohydrate groups of one glycoprotein molecule may crosslink with either amino group of lysine on another molecule. Disulfide exchange (linkage between free disulfide groups) is another common mechanism of aggregation through covalent bond formation. Noncovalent aggregates, on the other hand, can form through noncovalent interactions that occur between hydrophobic sites of adjacent protein molecules.¹⁰⁴ While exposure to the air–water interface has been proven to be a prelude to aggregation for many proteins such as hGH, BSA, LDH, and β -galactosidase, others such as t-PA and rhDNase are not as vulnerable.^{19,58}

Several factors influence protein surface adsorption and the amount of unfolding that occurs at an interface. One is the number and distribution of nonpolar amino acids on the protein surface, an important factor because adsorbed proteins predominantly expose their hydrophilic groups to water at the air–water interface.⁹⁹ Second is the protein rigidity/flexibility in solution as determined by the number of intrachain disulfide bonds. Hard proteins have more of the latter and are therefore less flexible than soft proteins. Protein flexibility strongly impacts the number of protein molecules that adsorb to the interface and their spreading rate.^{99,105} A

flexible, noncrosslinked protein will be able to unfold easier than will a highly structured and crosslinked one.

Figure 6 shows the in-process stability, as measured by size exclusion chromatography, for Des-phe human growth hormone (Des-phe hGH) spray dried with trehalose and different concentrations of Tween.¹⁰⁶ Low concentrations of Tween (below critical micelle concentration [CMC]) did not offer Des Phe-hGH significant protection against aggregation. On the other hand, the addition of 0.1% w/v Tween 20 provided higher in-process stability for Des Phe-hGH during spray drying, as shown by the lowest aggregate levels (soluble aggregates were measured). These results are consistent with those obtained by Mumenthaler et al.⁵⁸ Aqueous solutions of recombinant methionyl-hGH (Met-hGH) spray dried without surfactant showed significant aggregation after spray drying. Insoluble aggregates ranged from 4% to 4.7% and soluble (covalent and non covalent) aggregates were slightly over 20%. The addition of 0.1% w/v Tween 20 reduced the formation of aggregates to 5.4% (at an inlet temperature 90°C) and 7.9% (at an inlet temperature 150°C). A 0.1% w/v Tween 80 concentration was also reported to stabilize hGH against aggregation in solution.¹⁰³ Note that this concentration is much higher than the reported CMC value of Tween 80 (0.0013%).¹⁰⁷ Figure 7 shows that while

soluble aggregation is not eliminated by using surfactant, and is still at levels higher than using an atomization-free drying method (freeze drying), aggregation is greatly reduced. Atomization of excipient-free recombinant hGH (2 mg/mL)¹⁰⁸ resulted in 25% insoluble aggregates and 17.2% soluble aggregates. Atomization of the same solution with 0.05% Tween 20 resulted in <1% insoluble aggregates and 17% soluble aggregates. Similarly, spray drying with Tween 80 also led to the improvement in process stability of other proteins such as LDH⁶² and BSA.¹⁰⁹

There are two possible mechanisms by which nonionic surfactants protect proteins from the air–water interface. First, surfactant molecules displace protein molecules from the air–water interface perhaps by virtue of their lower molecular weight and hence faster diffusion to the air–water interface, accompanied by the fact that surfactant is favored at the surface at equilibrium too.^{58,62} Thus, less protein will migrate to the air–water interface and be susceptible to unfolding and aggregation. Additionally because surface tension is lowered after surfactant is adsorbed, there might be less damage to the protein that does adsorb at the air–water interface.¹¹⁰

The second mechanism of stabilization at the air–water interface involves binding to certain hydrophobic sites on protein molecules, and hence a decrease in intermolecular interactions that

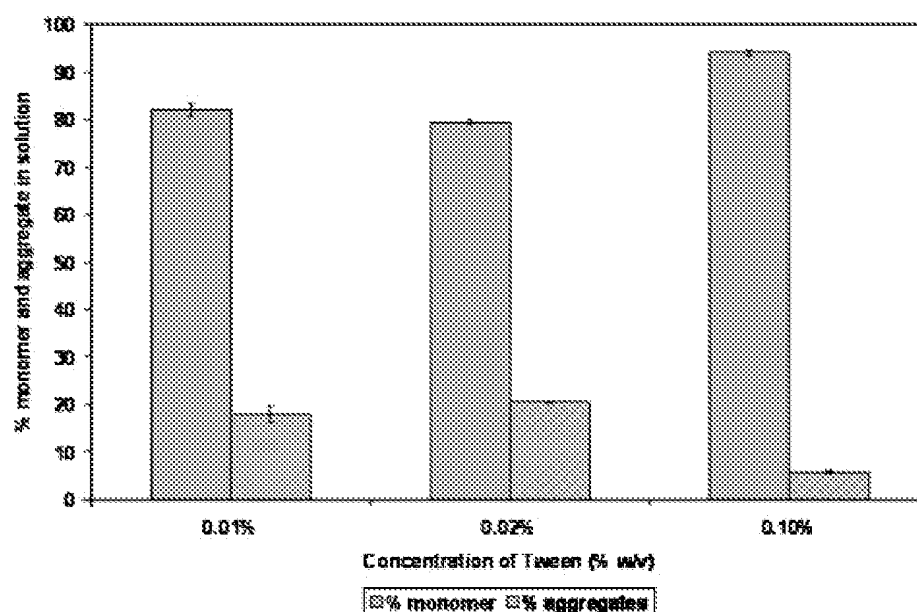


Figure 6. In-process stability of spray-dried Des Phe-hGH samples with different concentrations of Tween (a nonionic surfactant). SEC results showing percentage of soluble aggregate formation, as well as monomer content.

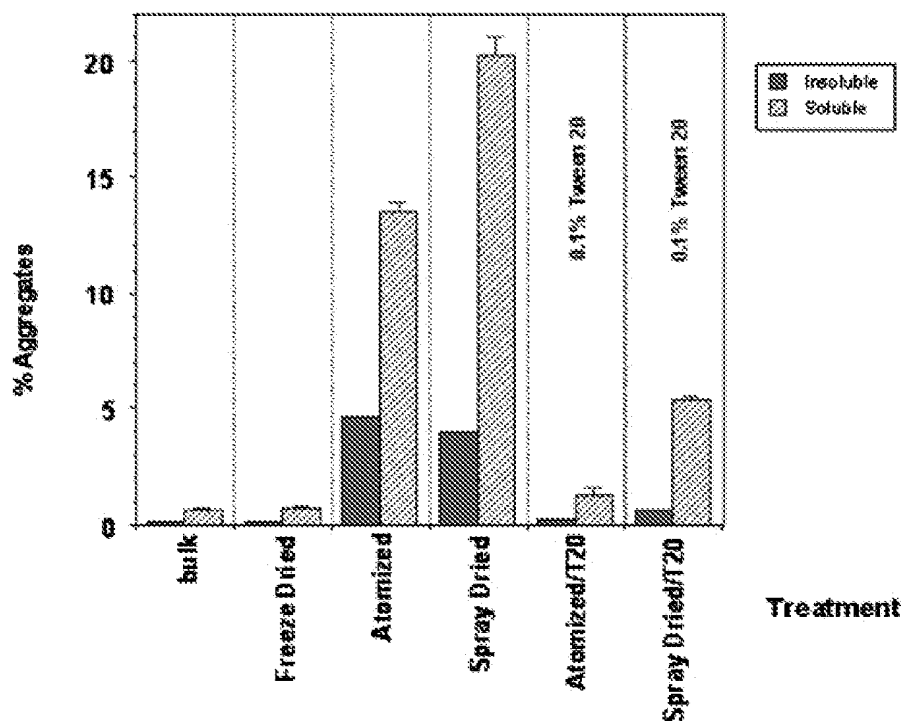


Figure 7. Spray drying of human growth hormone (hGH) 2 mg/mL hGH, 88 mM mannitol, 5 mM sodium phosphate, pH 7.8.⁵⁸ Freeze-dried results from Pikal, unpublished data.

would lead to aggregation. Katakam et al.¹⁰³ argue that because a surfactant forms a complete monolayer at the air–water interface at its CMC value, the protection it offers against aggregation should be related to its CMC value. In many cases, however, this was not the case. In cases of interaction, the concentration of surfactant needed to protect the proteins from aggregation at the air–water interface was correlated to the molar binding stoichiometry between surfactant and protein, rather than to the CMC of the surfactant. Values for molar binding stoichiometries for the different proteins studied showed only minor variations. For example, the molar binding stoichiometry of Tween 20 to HSA was determined to be ~11:1, to hGH was ~10:1 and to albutropin was ~10:1. The CMCs of Tween 20 (59 μ M) and Tween 80 (12 μ M) were found to shift to lower Tween concentrations in the presence of hGH.⁶⁴ On the other hand, the CMCs of both surfactants were shifted to >100 μ M in the presence of albutropin.¹⁰⁷ The authors attributed the latter observation to possible binding between surfactant and albutropin, and as a result, the amount of surfactant needed to saturate the air–water interface increased. Surfactants may bind to

the folded protein or to the partially unfolded protein. If surfactant binds to the native folded protein, then stabilization is explained by stating that the free energy of unfolding is increased by virtue of the binding interaction (i.e., the free energy of the native state is lowered by the binding interaction). In cases where surfactants bind transiently with partially un-folded protein molecules, they are believed to act as chemical chaperones that favor refolding over aggregation and sterically hinder intermolecular interactions that may lead to aggregation. Thus, the protein is prevented from falling into kinetic traps or more time is allowed for the protein to refold.⁶⁴

While nonionic surfactants generally do not denature proteins even at high concentrations,⁶⁴ ionic surfactants do. Ionic surfactants bind to proteins in two stages.^{100,111} The first involves electrostatic attraction forces (e.g., sulfate anion of sodium dodecyl sulfate (SDS) with positively charged lysine, histidine, and arginine) and binding of hydrophobic regions occur on both species by hydrophobic interaction. The second stage involves unfolding of protein tertiary structure followed by hydrophobic interaction with the exposed nonpolar groups of the protein.

Dehydration

Dehydration is the ultimate result of any drying method, and the extent of dehydration may differ from one drying method to another. The removal of 'bound water' from the system is accompanied by a reduction in the total number of intermolecular hydrogen bonds between water and protein molecules. Moreover, many charges on the protein molecules will be exposed to each other when water molecules of solvation are removed.¹¹²

According to the water substitution hypothesis,^{37,40,41,113,114} a significant thermodynamic destabilization occurs when the hydrogen bonding between protein and water is lost during drying. Therefore, native structure preservation can be accomplished by the use of a "water substitute" such as a saccharide or polyol lyoprotectant. A water substitute is a moiety that is capable of hydrogen bonding to specific sites at the surface of a protein much as water and stabilizes via a "thermodynamic mechanism." That is, stabilization is achieved by maintaining the free energy of unfolding very high such that essentially all of the protein is maintained in the native conformation. The relationship between preservation of native structure and protein long-term storage stability is based upon empirical observations and will be discussed later. Another mechanism proposed for stabilization during dehydration is a purely kinetic stabilization mechanism, whereby proteins are mechanically immobilized in a glassy solid matrix during dehydration.¹⁰ Translational and rotational motions of the protein, or motion of groups that constitute the protein, are too slow to allow significant unfolding during the drying operation.^{41,114} Thus, the protein conformation is stabilized regardless of the free energy of unfolding.

Nonreducing di- and tri-saccharides, such as sucrose, trehalose, or raffinose, are normally good drying stabilizers.^{37,40,41,113} They qualify as good water substitutes and also form glasses which, via hydrogen bonding, can couple protein dynamics to matrix dynamics.⁸⁶ Regardless of the mechanism, the key principle to insure protein stabilization during dehydration is that the 'water-substitute' or stabilizer remains in the same amorphous phase as the protein. Under certain conditions, some stabilizers will phase separate from the protein or crystallize from the amorphous phase during dehydration. For example, mannitol readily crystallizes out during freeze drying. Components that crystallize will not be available in the

amorphous phase to contribute to protein stabilization by either thermodynamic or kinetic mechanisms. Altering processing conditions and formulation components can manipulate the degree of crystallization.^{115,116} Under circumstances where mannitol remains mostly amorphous, mannitol has been shown to protect enzymes during lyophilization in a concentration-dependent manner.^{115,116} The presence of other components at appropriate levels can inhibit mannitol crystallization and thereby increase its availability as a stabilizer in the amorphous phase. For example, the presence of glycine and potassium phosphate has been shown to inhibit the crystallization of mannitol during freeze drying of hGH and LDH, respectively.^{81,115}

PHYSICO-CHEMICAL PROPERTIES CRITICAL TO STABILITY OF AMORPHOUS PHARMACEUTICALS

Drying can give rise to powders that are amorphous, crystalline, liquid crystalline, or a mixture of these phases.^{45,117–119} Many tools have been used to identify the physical state of the powder, most important of which are x-ray powder diffraction, modulated differential scanning calorimetry (MDSC), and polarized light microscopy.^{45,118}

Differences in physico-chemical properties and stability between amorphous formulations prepared by different drying methods have been observed, even when no phase separation occurred. However, the origins of these differences and their product quality consequences, particularly for long-term storage stability, are not always obvious. In this section, we will discuss some important physico-chemical properties that may be of particular value in predicting product quality consequences of drying method differences and hence assist in decision making with regards to drying method selection.

Protein Secondary Structure

Fourier transform infrared spectroscopy (FTIR) is an increasingly popular method for quantifying the loss of native structure or the degree of perturbation in secondary structure upon drying. Structural information is usually obtained by analysis of the amide I band located between 1600 and 1700 cm^{-1} . It arises mainly due to the in-plane C=O stretching vibration of the peptide

linkages that constitute the backbone structure and is known to be sensitive to protein secondary structures and conformational changes.¹²⁰ Others have also used the amide III region for the quantitative analysis of secondary structure, for example, in secondary structure assessment of tetanus toxoid¹²¹ and recombinant human albumin.¹²² There are several ways for quantitative analysis of the area-normalized, baseline-corrected second-derivative spectra at the amide I (or III) regions to measure structural damage, as for example determining the spectral correlation coefficient (r value). The latter is a value that measures the similarity or the area of overlap between second-derivative amide I spectra in solution (native state or reference) and in the solid state.^{123,124}

In-Process Stability

As was discussed earlier, dehydration is the main contributor to the perturbation in protein secondary structure during drying.¹²³ Several studies document a relationship between the drying method and preservation of natively protein structure. The degree of perturbation of protein secondary structure can differ from one drying method to the other due to the nature of stresses that occur during drying (other than dehydration). Such stresses can damage native protein structure to different degrees. Allison et al.¹⁸ reported that less sucrose was required to stabilize the native structure of actin in air drying, as compared to freeze drying. Crystallization of one component or separation of two or more amorphous phases (i.e., phase separation) during drying may also result in unfolded protein in the dried powder.^{44,48,119,125,126} A freeze-dried PEG/dextran system showed phase separation, which had a negative impact on the secondary structure of hemoglobin in the dry solid.¹²⁷ Moreover, it was found that annealing in the frozen state accelerated phase separation and caused more perturbation in protein secondary structure, the magnitude of the effect increasing with increasing annealing time.⁹⁷ The use of a different drying method, SFD, led to a more native protein structure.⁷⁰ Recall that freezing rate in SFD is orders of magnitude higher than in a normal freeze drying cycle. It is possible that the fast cooling rate, and hence quenching the system in a frozen state, occurred at a timescale much faster than the timescale required for phase separation.⁷⁰

Although the expected outcome of more native-like protein structure is better in-process stability (or recovery of biological activity post drying), there are conflicting reports as to whether there is a correlation or not. It is possible that this uncertainty arises because activity retention might be correlated with reversibility of denaturation (which differs from one protein to another). In other cases, the relationship between in-process stability and secondary structure might simply be obscured by a lack of sufficient sensitivity of FTIR to the structural changes that impact stability.¹²⁸ A few examples on the influence of drying method on protein secondary structure and in-process stability are documented. In a study by DePaz et al.,³² structural perturbation of subtilisin was found to be greater in excipient-free lyophilized formulations versus spray-coated formulations which correlated well with in-process stability. On the other hand, a poor correlation was observed between preservation of native structure and recovery after drying in a series of samples generated using different drying methods in both the presence^{18,32} and absence¹⁸ of stabilizers.

Storage Stability

The preservation of native protein structure during and after drying is expected to strongly influence the storage stability of proteins.^{40,41,128} There are few studies, however, that correlate the degree of preservation of secondary structure in the solid state to storage stability. One such report is by Prestrelski et al.¹²⁹ Storage stability of freeze-dried Interleukin-2 (IL-2) formulations at both 30 and 45°C improved dramatically as the protein acquired a more natively structure after drying, evidenced by higher spectral correlation coefficients (r) (Fig. 8). Protein freeze dried from solutions formulated at a higher pH showed a greater degree of perturbation in secondary structure and, as a result, poor stability. Also Chang et al.¹³⁰ found a correlation between sucrose level, perturbation of secondary structure after drying and storage stability of freeze-dried formulations of IL-1 receptor antagonist.

The correlation between storage stability and protein secondary structure of systems prepared by different drying methods, on the other hand, is poorly understood. Sane et al.¹⁷ studied the secondary structure of a monoclonal antibody in spray- and freeze-dried formulations by Raman spectroscopy. The authors found similar secondary structure of the protein in spray- and

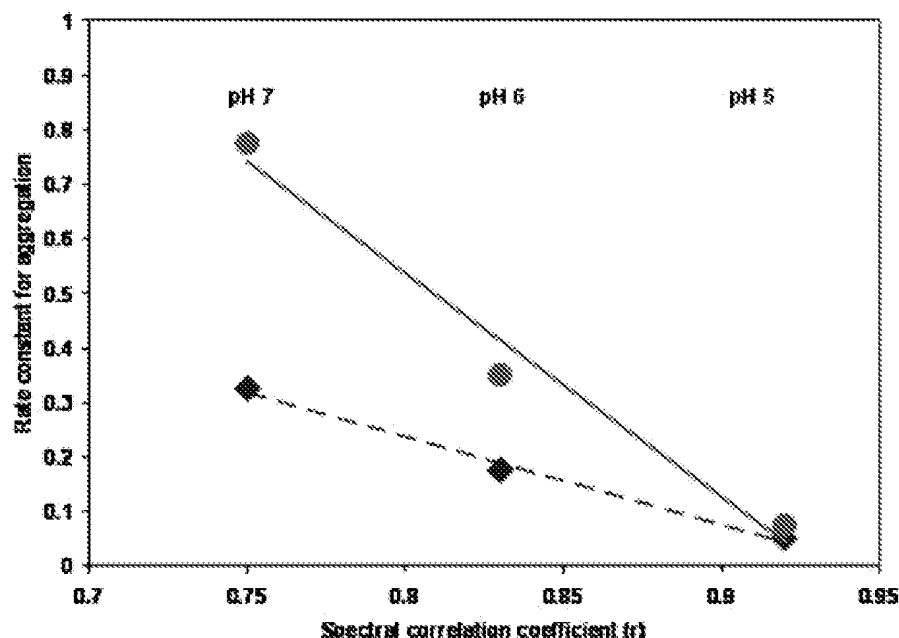


Figure 8. Rate constants for aggregation of interleukin 2 (IL-2) formulations freeze dried from different pH at both 30°C (solid diamonds) and 45°C (solid circles) as a function of protein native structure.

freeze-dried formulations. In spite of these similarities, storage stability in the spray-dried formulation was inferior. Changes in other powder properties that may help explain stability differences, however, were not examined.

Molecular Mobility

Little has been done to measure and compare molecular mobility in the same formulation prepared by different drying methods. The two major types of molecular mobility investigated have been global dynamics (motions that occur on very long timescales and are correlated or “coupled” to viscosity) and fast dynamics (motions that occur on very short timescales and involve small length scales). The impact of molecular mobility on the storage stability of proteins in the glassy state prepared by different drying methods is not well studied in the literature. In this section, we will discuss the importance of molecular mobility and its impact on storage stability.

Global Dynamics (α -Relaxations)

Upon cooling a melt, volume, enthalpy, and entropy decrease, and viscosity is greatly in-

creased, thereby slowing molecular motion such as translational or rotational diffusion.^{113,131} Motions strongly correlated to viscosity are termed α -relaxations or global motions, with the term “structural relaxation time” denoting the time constant for a fundamental “ α ” or global motion.^{113,131,132} In a glass, with more limited “free volume,” or space in which a molecule can easily move, whole molecule motion requires simultaneous motion of many neighbors, or motion that is highly cooperative.¹³¹ Since highly cooperative motion is improbable, and therefore occurs only infrequently, the time taken for molecules in the system to undergo global motion (i.e., motion on a large length scale such as over a molecule diameter) will, therefore, increase.^{131,133,134} The reciprocal of structural relaxation time ($1/\tau$), may be termed a “global mobility.”

Structural relaxation time can be measured by measuring the time-dependence of enthalpy relaxation using calorimetry. Global (or α -relaxations) in glasses typically occur over a time period in excess of 100 s, and τ values of hours and even years are not uncommon at pharmaceutically relevant temperatures.^{113,134,135} At temperatures well below the glass transition temperature (T_g), α -relaxations become very highly cooperative and

slow greatly.^{45,113} The Kohlrausch-Williams-Watts (KWW) equation (Eq. 1) is commonly used to describe the kinetics of relaxation as obtained from calorimetric techniques.^{35,45,113,134}

$$\Phi(t) = \exp \left[- \left(\frac{t}{\tau} \right)^\beta \right] \quad (1)$$

where $\Phi(t)$ is the relaxation function and t is time. τ is the characteristic relaxation time and β ($0 < \beta \leq 1$) is the stretching parameter,^{45,113,136} which reflects the extent to which the relaxation process deviates from exponential behavior.^{134,135}

Relationship between α -Relaxations and Reactivity. Although the high viscosity in a glass ($>10^{12}$ Pa·s) greatly slows the α -relaxations,^{137,138} many reactions do proceed at a measurable rate below T_g ,^{134,135,137,139–147} whether the reaction is unimolecular or bimolecular,¹⁴⁷ for example, chemical reactions or aggregation.¹⁴¹ The fact that these reactions occur means there is sufficient molecular mobility and free volume in the amorphous state to allow the acquisition of molecular configurations favorable for a chemical or physical reaction (or both) to occur.^{134,135,141–146,148–150} Since both degradation and structural relaxation in the solid state require motion of some type, it is expected that storage stability and structural relaxation are correlated.^{113,143,145,148} Moreover if the motion required for a certain degradation reaction to occur requires the same type of mobility as the α -relaxations, then structural relaxation time and stability should be nearly proportional. It is possible to evaluate the effect of molecular mobility on degradation in amorphous pharmaceuticals by comparing the degradation rates with structural relaxation times.¹³⁵ A perfect coupling between both would result in direct proportionality. One, however, should not generally expect a direct proportionality between stability and structural relaxation time since the free volume requirement for decomposition may not be exactly the same as that for structural relaxation.¹³²

Good correlations have been reported between chemical reaction rates and structural relaxation time for low molecular weight drugs,^{132,140,151} as well as for peptides and proteins.¹³⁹ Guo et al.¹⁴⁰ reported the similarity of temperature dependence for the rate of chemical decomposition (cyclization) of quinapril below T_g and τ (estimated using Adam-Gibbs-Vogel equation), suggesting that cyclization of quinapril is related to molecular mobility. A similarity of temperature dependence

between t_{90} (the time for 10% of the drug to undergo a degradation reaction) for an acetyl transfer reaction (between aspirin and sulfadiazine) and τ below T_g in amorphous matrices with dextran and polyvinyl pyrrolidone was also reported by Yoshioka et al.,¹⁵¹ suggesting that t_{90} for acetyl transfer was related to molecular mobility. Roy et al.¹³⁹ determined a good correlation between chemical degradation rates of lyophilized formulations of a Vinca alkaloid-antibody conjugate with $T-T_g$ at various moisture contents and at two different temperatures. Similar to chemical reactions, good correlations have been reported between rates of physical changes and structural relaxation time for peptides and proteins. Duddu et al.¹⁵² found a correlation between aggregation of a monoclonal antibody in lyophilized formulations and molecular mobility, as measured by enthalpy relaxation time. Also, Yoshioka et al.¹⁵³ found that aggregation of BSA in the solid state increased as molecular mobility (measured by ^1H nuclear magnetic resonance) increased.

Relationship between 'Annealing' a Glass and Improving its Storage Stability. Slowing α -relaxations should minimize the reactivity of a species in the amorphous state.¹⁴¹ The spontaneous process by which different properties of a glass stored below its T_g continue to change over time, in an attempt to attain equilibrium, is called physical aging.¹⁵⁴ For example, free volume decreases, structural order increases (i.e., configurational entropy decreases) and heat is given off (enthalpy relaxation).^{155,156} Physical aging has been shown to slow α -relaxations in glasses, thereby increasing τ .^{140,141} Physical aging can be accomplished by several means, for example, by subjecting a glass to high pressure isothermally¹⁵⁷ or by annealing—otherwise also referred to as 'densification'.^{143,148,158–160} The term 'annealing' simply means heating an amorphous sample below its T_g for a period of time. A glass will approach the "equilibrium glassy state" asymptotically during an annealing process. In theory, this should result in an increase in the structural relaxation time (i.e., a decrease in mobility).^{143,148,158,159} This, in turn, should slow a degradation process.^{132,143} The earliest report on the possible beneficial effect of annealing on the glassy state is by Mardaleishvili and Anisimov.¹⁶¹ In their study, polymethyl methacrylate (PMMA) films (with an initiator for formation of free radicals via a photochemical

reaction) were annealed at two different temperatures below T_g for different periods of time. This was followed by sample irradiation at a wavelength of 365 nm to initiate free radical formation. Accumulation of free radicals in the different films was measured as a function of time. The sample annealed at the higher temperature showed the smallest rate of accumulation of free radicals. Additionally as annealing time at the same temperature increased, the rate of accumulation of free radicals decreased. In all cases, the rate of free radical formation was observed to decrease with time. Madsen et al.¹⁶⁰ followed the stability of annealed and untreated borophosphosilicate glass films. Degradation via loss of boron (B) was followed by observing the ratio of B-O to Si-O peak intensities using FTIR. High temperature annealing resulted in restructuring and ordering of the glass leading to improved long-term chemical stability. A 2-year storage period revealed more significant changes in untreated films than in annealed films. Untreated films showed hazing (opalescence) and a decrease in height of the B-O peak, and hence a decrease in the ratio of B-O to Si-O peaks. Annealed films showed no hazing and the ratio of B-O to Si-O peak heights remained unchanged indicating no significant loss of B during prolonged storage. Recently, Hill et al.¹³⁷ investigated the Maillard reaction in an annealed and untreated glassy system of lysine HCl in trehalose, sucrose, and glucose (in a ratio of 13:5:1, respectively). They found that aging moderately lowered the reaction rate (~20%).

Process Implications. In summary, evidence from the literature shows that thermal history, α -relaxation, and annealing all impact global dynamics. The latter has been correlated to storage stability. Therefore, one can reason that if largely different thermal histories and/or annealing conditions are involved in different drying processes (revisit and compare Figs. 1–5), there may well be significant differences in global dynamics between glasses prepared by different methods and hence different storage stability. It was reported¹⁶² that a sucrose glass prepared by spray drying has a smaller relaxation time (i.e., faster global mobility) than quench-cooled sucrose and freeze-dried sucrose. However, to this date, there are still insufficient data in the literature to define the impact of different drying processes on both global dynamics and storage stability in the same system.

Fast Dynamics (β -Relaxations)

It has been proposed that dynamics other than global motions may be important for protein stabilization in glasses, since faster relaxations termed β -relaxations have been observed in glassy materials.^{141,163,164} β -relaxations are representative of fast dynamics and they involve localized motion of specific portions of the molecule.¹⁶⁵ They can occur over a broad range of timescales, anywhere between vibrational and rotational motion timescales.^{141,163,164,166} It is argued that β -relaxations influence diffusion of small molecules such as gases in systems of larger molecules, and possibly control small amplitude protein motions that can result in protein aggregation.^{141,163,164} The concept of fast dynamics is relatively new to the pharmaceutical industry.

Motivation for the Study of Fast Dynamics. Where an increasing number of studies have demonstrated that storage stability of amorphous pharmaceuticals is closely related to global dynamics, there are a few examples in literature that suggest a poor correlation between stability and global dynamics. The contribution of molecular mobility was found to be small in insulin degradation and dimerization in formulations freeze dried with trehalose under high (but not low) humidity conditions,¹⁶⁷ as well as in poly(vinyl)pyrrolidone (PVP) systems.^{167,168} Excellent preservation of enzyme activity was observed for EcoRI enzyme in sugar–enzyme systems above the glass transition temperature (sugars were sucrose, trehalose, or raffinose) dried by vacuum drying.¹⁶⁹ Similar results were obtained above the glass transition temperature of formulations of the restriction endonuclease HindIII enzyme in sucrose or trehalose matrices with glycerol,¹⁶⁹ and formulations of glucose-6-phosphate dehydrogenase in a sucrose matrix.¹⁷⁰ This is a surprising observation since above T_g , global motions and diffusion are nominally sufficiently free to allow relatively rapid reaction rates.

It has been suggested that well below the glass transition temperature, the contribution of global motions is minimum in reactions (chemical or physical), and the degradation process is controlled mainly by β -relaxations.^{141,163,164} Hence, global dynamics may not always be critical in storage stability problems.

Correlation of Fast Dynamics to Stability. Recently, incoherent elastic neutron scattering using

the high flux backscattering spectrophotometer (HFBS) has been used to measure fast dynamics in the solid state. A comprehensive review of the basis of operation of HFBS and details of data analysis are reported in literature.^{163,171} Briefly in an incoherent elastic neutron scattering experiment, monoenergetic incident neutrons collide primarily with hydrogen atoms in the samples. After collision, neutrons are scattered elastically or inelastically. The intensity of the scattered neutrons, I_{inc} , is measured as a function of the scattering wave vector (Q) and frequency (ω). Scattering events from motions at timescales of >5 ns or more are counted as elastic with the HFBS, and static structural information on the sample is obtained. The Q -dependence of the incoherent elastic scattering I_{inc} is analyzed in terms of the Debye-Waller factor (a harmonic oscillator model) where the mean-squared amplitude of atomic motions in the sample $\langle \mu^2 \rangle$ is given by

$$I_{\text{inc}}(Q, \omega = 0) \propto \exp\left(-\frac{1}{3}Q^2\langle \mu^2 \rangle\right) \quad (2)$$

Cicerone et al.¹⁴¹ showed that the addition of a small amount of glycerol to an amorphous matrix of horse radish peroxidase (HRP) and PVP resulted in increased stability of HRP, even though glass transition temperature was lowered. A similar effect was observed with yeast alcohol dehydrogenase. These results are surprising given the fact that glycerol causes a decrease in T_g which would decrease molecular mobility on the global motion timescale. Improvements in stability were consistent with a decrease in mobility associated with β relaxation, as measured by neutron scattering studies. Therefore it appeared that glycerol, a plasticizer for global motions, acted as an anti-plasticizer for the β -motions.

Recently, Yoshioka et al.¹⁶⁶ used ^{13}C solid-state NMR to study the correlation between storage stability and β -relaxations of freeze-dried insulin formulations, using $T_{1\rho}$ (rotating frame spin-lattice relaxation time) as a measure of mobility. $T_{1\rho}$ reflects rotational motion of protons with a certain relaxation time value (τ_c).¹⁷² Yoshioka et al. found that the degradation rate of insulin freeze dried with dextran was greater than insulin freeze dried with trehalose at low relative humidity. Insulin degradation occurs via a cyclic intermediate formation, which is not believed to require large-scale motions.¹⁶⁶ Improvement in stability with trehalose was attributed to the inhibition of

β -relaxations, as evidenced by higher $T_{1\rho}$ values with trehalose formulations (~ 200 ms in trehalose vs. ~ 100 ms in dextran), possibly as a result of the greater ability of trehalose to interact with insulin via hydrogen bonding.

Process Implications. The effect of thermal history on β -relaxations is not well understood but evidence from the literature has shown that formulation does impact fast dynamics. Furthermore, in some cases, fast dynamics has been correlated to storage stability.^{141,163,164,166} If thermal history does in fact impact fast dynamics in systems where fast dynamics are relevant to stability, then one would expect process variations would impact stability.

Specific Surface Area (SSA) and Surface Composition

Motivation for the Study of SSA and Surface Composition

In the studies by Sane et al.¹⁷ mentioned earlier, no differences were observed between the secondary structure of rhuMAb formulations prepared by spray drying or freeze drying. However, storage stability of rhuMAb was better in freeze-dried formulations. It is well known that powders produced by spray drying usually have a higher SSA than corresponding materials prepared by freeze drying. Therefore, we expect spray-dried rhuMAb formulations to have higher SSA. Moreover, because of its surface activity, we expect that more rhuMAb has migrated to the surface of the spray-dried powder. It is becoming well known that there may be significant separation of protein from stabilizer during drying. The surface region becomes rich in protein and the interior therefore must become richer in stabilizer (Fig. 9). Although there may be spatial variation in composition in freeze-dried materials as well, it does appear that composition variation in spray-dried materials can be an order of magnitude larger.^{16,173} Since one normally finds that the stability of a given protein improves as the weight ratio of stabilizer to protein increases, it follows that the separation of components may have an adverse stability consequence. That is, the protein near the surface would be much less stable than the overall composition would suggest. However, the implications of such component separation on storage stability are poorly understood. Determination of surface composition of dried powders and correlating these measurements to

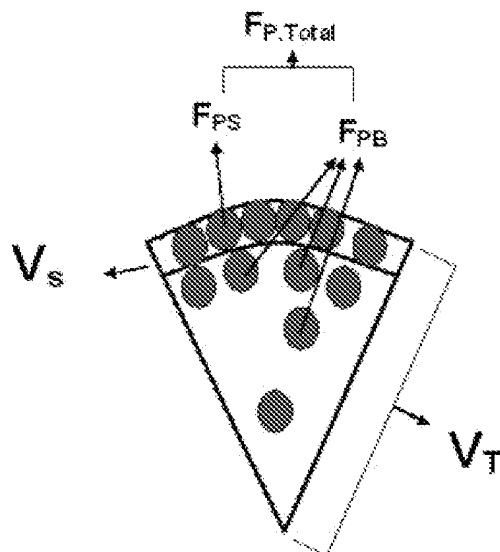


Figure 9. Cross-section through a dried particle where active ingredient (protein) molecules (closed circles) are not homogeneously distributed throughout the stabilizer matrix. V_S = Volume fraction of the surface detected by ESCA; V_T = Total volume of the particle; F_{PS} = Fraction of protein at the surface detected by ESCA; F_{PB} = Fraction of protein in the bulk of the dried particle; and $F_{P,Total}$ = Total amount of protein added ($F_{PS} + F_{PB}$).

SSA measurements is a relatively new application in the pharmaceutical field. In this section, we will discuss the importance of surface composition and SSA to stability of proteins and biologics.

Composition Heterogeneity

Composition or chemical heterogeneity refers to the separation of chemical components during drying without phase separation.^{16,62,109,174–177} That is, separation of stabilizer from protein can occur, and hence there will be a concentration gradient of the protein molecule across the dried particle, and hence uneven distribution of protein and excipients (Fig. 7). Composition heterogeneity is thought to arise from the surfactant properties of the protein (meaning that protein thermodynamically favors migration to the air–water interface),^{16,62,109,175–177} and the use of a surfactant has shown to significantly reduce the accumulation of protein at the surface, especially for spray-dried powders.^{16,62,109,175} Development of composition heterogeneity from a solution initially uniform in composition requires molecular mobility. That is, one must

have the molecular mobility to support the mutual diffusion needed to create separation of components. Such composition heterogeneity could arise during freezing for a freeze drying process and during most of the drying process for a spray-dried material. However, once the material is well below its glass transition temperature, it seems unlikely that sufficient translational mobility would exist to allow further separation of components.

Component separation and surface composition of dried pharmaceutical powders have been documented by using special surface analysis techniques such as Electron Spectroscopy for Chemical Analysis (ESCA) or x-ray photoelectron spectroscopy (XPS).^{16,62,109,174–176,178,179} Briefly, surface analysis by ESCA is accomplished by irradiating the solid sample in vacuum with monoenergetic soft x-rays and sorting the emitted electrons by energy.¹⁸⁰ The spectrum obtained is a plot of the number of emitted electrons per energy interval versus their kinetic (binding) energy. Since the mean free path of electrons is very small, electrons emitted from deeper layers lose energy by inelastic collisions on the way to the surface and are no longer able to leave the solid phase. Therefore, electrons which are detected originate only from the top few atomic layers, with a sampling depth of ~ 50 Å below the surface.^{16,180}

Surface Coverage

The spectral peaks from a mixture are approximately the sum of the elemental peaks from the individual constituents. From an analysis of the relative amount of the different elements in the pure components and in the powder, one can determine surface composition of each chemical component (i.e., surface coverage or surface concentration for each component).^{62,109,178,179,181} ESCA can detect all elements except helium and hydrogen atoms.¹⁸⁰

Assume a freeze-dried powder composed of a protein such as BSA, sucrose and Tween. BSA mainly contains carbon (C), oxygen (O), and nitrogen (N). Both the stabilizer and surfactant contain only C and O. The N peak is, therefore, indicative of the presence of the protein in the 50 Å surface region^{16,179} of the freeze-dried powder, and therefore it is possible to determine the protein surface coverage.^{62,109,174–176,178} The atomic percent of element (n) in the pure chemical component (i.e., molecule), j (i.e., I_j^n) are designated I_p^n for the protein, I_T^n for the surfactant

Tween 20, and I_S^j for the stabilizer. For each of the elements C, O, N in the powders, the relative amount can be expressed as:¹⁷⁹

$$I_{\text{Sample}}^{\text{C}} = I_{\text{P}}^{\text{C}} \cdot \gamma_{\text{P}} + I_{\text{T}}^{\text{C}} \cdot \gamma_{\text{T}} + I_{\text{S}}^{\text{C}} \cdot \gamma_{\text{S}} \quad (3)$$

$$I_{\text{Sample}}^{\text{O}} = I_{\text{P}}^{\text{O}} \cdot \gamma_{\text{P}} + I_{\text{T}}^{\text{O}} \cdot \gamma_{\text{T}} + I_{\text{S}}^{\text{O}} \cdot \gamma_{\text{S}} \quad (4)$$

$$I_{\text{Sample}}^{\text{N}} = I_{\text{P}}^{\text{N}} \cdot \gamma_{\text{P}} + I_{\text{T}}^{\text{N}} \cdot \gamma_{\text{T}} + I_{\text{S}}^{\text{N}} \cdot \gamma_{\text{S}} \quad (5)$$

where $I_{\text{Sample}}^{\text{C}}$, $I_{\text{Sample}}^{\text{O}}$, and $I_{\text{Sample}}^{\text{N}}$ are the atomic % of C, O, N, respectively, on the sample surface being analyzed by the X-ray beam (which is the surface region roughly 50 Å deep, and is the parameter provided by the apparatus from the signal intensity and the known sensitivity factor for that element). In summary, assuming that the elemental composition in the surface region is a linear combination of the elemental compositions of the different molecular species present, the data on the elemental composition can be used to estimate the molecular composition of the surface layer by solving a matrix equation: $\mathbf{C}\gamma = \mathbf{c}$, where \mathbf{C} is the matrix containing the elemental compositions of the molecular species (atomic %), \mathbf{c} is the vector containing the elemental surface composition (atomic %) and γ is the surface coverage of the different molecular species.¹⁷⁴ Note that since surfactant and stabilizer molecules are devoid of N atoms, $I_{\text{T}}^{\text{N}} = I_{\text{S}}^{\text{N}} = 0$. γ_j is the relative coverage of component “j,” that is, γ_{P} , γ_{T} , and γ_{S} are the fractions of the surface area covered with protein, surfactant (Tween), and stabilizer, respectively. In reality since some signal could originate from slightly below the surface itself, it is probably more proper to interpret γ_j as the volume fraction of the component j in the surface region being sampled by the X-ray beam. The linear least squares method is then applied to solve the matrix for each γ_j . Note that the relationship given by the equations relating I_{Sample}^j to γ_j is based on the assumption that the ESCA response from a given component is proportional to the fraction of the surface area (or volume in the surface region) that is occupied by that component.

Factors Affecting Protein Surface Coverage

The surface activity of different components has shown to strongly impact protein surface coverage. The surface composition of spray-dried protein–lactose systems was studied by ESCA.¹⁷⁸ Lactose–protein solutions were prepared in different ratios, and in all cases, the ratio of BSA or

sodium caseinate to lactose on the powder surface was always higher than would be expected from a homogeneous system. Solution surface tension measurements prior to spray drying showed that surface tension decreased as protein concentration increased, with a plateau level attained at a protein concentration of ~1%. ESCA measurements revealed that protein starts to appear on the powder surface created by drying solutions containing as little as 0.01% protein. As protein:lactose ratio increased, a sharp increase in protein surface coverage in the dry powder was observed. At a protein:lactose ratio of 5:95, roughly 65% of the surface was covered with protein and a maximum protein surface coverage of 75% was attained. Similarly, Adler et al.¹⁰⁹ detected a surface excess of BSA on the surface of spray-dried powder formulated with trehalose (i.e., more protein than would be expected from the surface of a homogeneous sample). On the other hand, a surface deficiency of BSA was observed upon the addition of Tween 80 or SDS to the BSA/Trehalose system (i.e., less protein than would be expected from the surface of a homogeneous sample). Similar results were obtained with spray-dried LDH/trehalose systems, in the presence and absence of Tween 80.⁶² Therefore, it appears that composition heterogeneity occurs during drying, and that the most surface active component in solution (assumed to be protein in surfactant-free formulations and surfactant in surfactant-rich formulations) enriched the surface of the drying solutions and hence was concentrated on the surface of the dried powders.^{27,62,109,175,176,178,182} Other studies confirm that the composition at the droplet surface was preserved during spray drying.^{176,183} In contrast, in a mixture of glycine and lactose, neither component is surface active and neither component showed any accumulation at the surface.¹⁷⁸

Diffusion and convection are the rate-limiting steps for adsorption and mass transport during spray drying.^{27,182,183} Consequently, surface active molecules with fast adsorption kinetics and fast diffusion (i.e., small molecular weight) are likely to be adsorbed faster than slower moving, even if the latter could provide a lower equilibrium surface tension.^{27,62,109,182} Here, adsorption refers to the migration of protein and/or surfactant molecules to the air–water interface. Landstrom et al.¹⁸³ studied the competitive adsorption behavior between BSA (molecular weight: 66000) and β -lactoglobulin dimer (β -Lg)

(molecular weight: 36000) in solution and their surface accumulation in spray-dried powders. Both proteins are surface active, with β -Lg showing more surface activity than BSA. Additionally, diffusion coefficient (D) of β -Lg in solution ($9.7 \times 10^{-11} \text{ m}^2/\text{s}$) is higher than that of BSA ($6.7 \times 10^{-11} \text{ m}^2/\text{s}$). At various proportions of proteins used in solution, β -Lg was always found to dominate the surface of spray-dried powders, although it is not obvious whether the dominant effect was differential diffusion or differential surface activity.

The drying method, the type of interactions between a stabilizer and protein and the propensity of a stabilizer to crystallize out impact protein surface coverage. In a study by Fureby et al.,¹⁷⁶ porcine trypsin was freeze dried with different stabilizers (lactose, sucrose, mannitol, α -cyclodextrin, and dextrin), and protein surface coverage was measured by ESCA after drying. The observed differences in protein surface coverage were correlated to differences in interactions between the protein and the different stabilizers used (e.g., chelation vs. no chelation), and propensity of some stabilizers to partially crystallize out (mannitol and sucrose). When frozen solutions were subjected to an annealing treatment above T_g ,¹⁷⁵ protein surface coverage increased (except in one case with α -cyclodextrin, suspected to chelate with the protein). Upon spray drying the

formulations, protein surface coverage was significantly higher than the corresponding freeze-dried formulations.¹⁷⁶

The drying method and molecular weight of the stabilizer used were also shown to impact protein surface coverage.¹⁸⁴ BSA was freeze dried and spray dried with stabilizers of different molecular weight (trehalose, raffinose, stachyose, dextran 10000, dextran 40, and ficoll 70). Protein surface coverage was measured by ESCA after drying. Similar to the results obtained by Fureby et al.,^{175,176} protein surface coverage was significantly higher in spray-dried preparations as compared to the same preparations freeze dried (Fig. 10). Additionally, protein surface coverage was found to be a function of molecular weight of the stabilizer. More protein surface coverage was observed with higher molecular weight stabilizers, regardless of the drying method (Fig. 10). Similar observations were obtained with myoglobin freeze-dried formulations.¹⁸⁴ If differential diffusion coefficient were controlling surface concentration, one would expect greater surface protein concentration in the case of small molecular weight excipients (i.e., larger difference in diffusion coefficients between excipient and protein). However, surface protein concentration was larger with the polymeric excipients indicating that there are other factors that may control protein surface coverage.

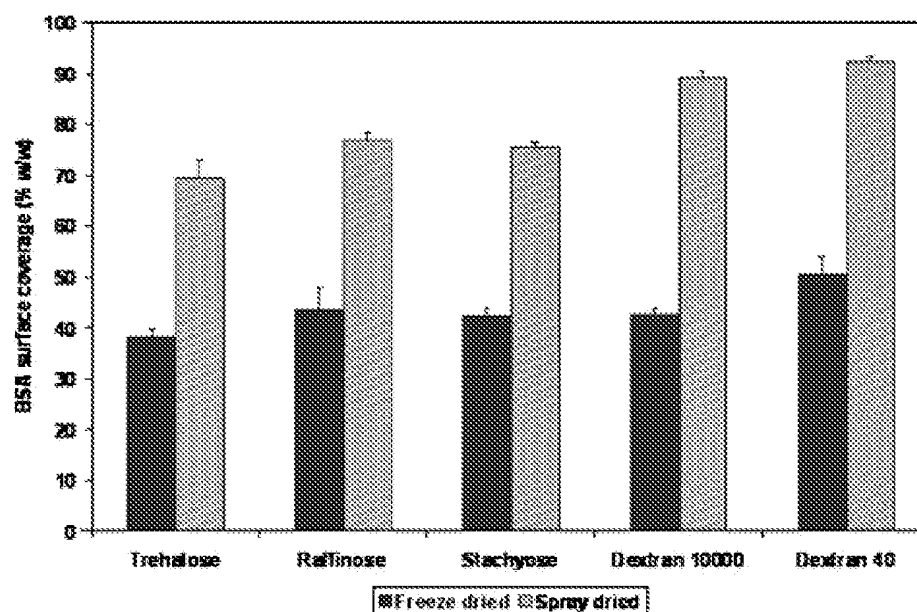


Figure 10. Protein surface coverage for freeze- and spray-dried formulations of BSA with stabilizers of different molecular weight. Composition of all formulations is 5% BSA and 95% stabilizer.¹⁸³

Total Protein Surface Accumulation and Factors Affecting Total Protein Surface Accumulation

Total protein surface accumulation ($\%P_{\text{total-surface}}$) reflects how much of the total protein (% w/w) added to the formulation has accumulated at the surface of the powders. Total protein surface accumulation is an important parameter since it is the protein fraction in a potentially reactive state. For long, ESCA studies have traditionally only reported protein surface coverage. The latter is a good measure of the degree of homogeneity only at the surface but does not directly measure the amount of protein at the surface. Using both protein surface coverage and SSA, one can calculate what fraction of the total protein has accumulated at the surface of the powder and what fraction remains in the bulk. If SSA is very low the fraction of protein accumulated at the surface will be small even if protein surface coverage is high.

Total protein surface accumulation can be calculated from knowledge of the protein surface coverage, the volume fraction of the powder sensed by ESCA, and the SSA of the powder. The volume fraction sensed by ESCA can be given by:

$$\frac{V_S}{V_T} \quad (6)$$

where V_S is the volume of the outmost shell and V_T is the total volume of particles. V_T is mass divided by density (m/ρ) and V_S is unit area (A) times length (l) where $l = 50 \text{ \AA}$ or 5 nm , that is, the thickness of the outermost detected shell by XPS. Since SSA is area per unit mass (m^2/g), then V_S can also be expressed as $l \times m \times \text{SSA}$. Therefore,

$$\frac{V_S}{V_T} = \left[\frac{l \times m \times \text{SSA}}{M/\rho} \right] = l \times \text{SSA} \times \rho \quad (7)$$

where ρ has been taken to be roughly 1.1 g/cm^3 .²⁷ Therefore, total protein surface accumulation or

the percent of total protein occupying the surface ($\%P_{\text{total-surface}}$) can be given by:

$$\%P_{\text{total-surface}} = \frac{F_{\text{SP}} \times (V_S/V_T) \times 100}{F_{\text{PTotal}}} \quad (8)$$

where F_{SP} is the fraction of the surface that is protein (i.e., $\gamma_P/100$) and F_{PTotal} is the total fraction of the formulation that is protein.

Calculations for $\%P_{\text{total-surface}}$ from data in the literature, in the few cases where SSA and ESCA data were both available, strongly suggest that both drying method and the presence of surfactant do impact total protein surface accumulation! A summary for data analysis from a study Yu et al.¹⁷⁷ is presented in Table 1. Lysozyme and trehalose formulations with and without Tween 20 were prepared by SFD and spray freezing into liquid nitrogen followed by freeze drying (SFL). Higher $\%P_{\text{total-surface}}$ was observed with SFD powder, consistent with its highest SSA and high protein surface coverage. In contrast, the SFL powder with surfactant had the lowest $\%P_{\text{total-surface}}$, consistent with its lowest SSA and very low protein surface coverage.

Relationship between Total Protein Surface Accumulation, Drying Method, and Storage Stability

If one assumes that the measured or observed rate constant for a degradation reaction (k_{obs}) is a summation of the contribution of stability from both the surface and bulk protein, one may write:

$$k_{\text{obs}} = k_S F_{\text{PS}} + k_B F_{\text{PB}} \quad (9)$$

where k_S is the rate constant for decomposition of surface protein, k_B is the rate constant for decomposition of bulk protein and F_{PB} is the fraction of bulk protein. Thus, in a formulation with a large protein surface excess the contribution of k_S could be sufficiently large that surface protein dominates stability behavior.

Table 1. Chemical Heterogeneity with a Formulation of Lysozyme (5 mg/mL) and Trehalose (100 mg/mL)

Drying Method	0.1% Tween	SSA (m^2/g)	Protein Surface Coverage (% w/w)	Total Protein Surface Accumulation ($\%P_{\text{total-surface}}$)
SFL	No	40.9	13.20	62
	Yes	26.9	7.34	23
SFD	No	51.1	17.15	>99
	Yes	54.4	5.75	36

Formulations with surfactant contained 1 mg/mL Tween 20 (T20). Formulations were prepared by spray freeze drying (SFD) and by spray freezing into liquid nitrogen followed by freeze drying (SFL). Data from Yu et al.¹⁷⁶

Based on formulation composition, lysozyme surface coverage would be $\sim 5\%$ w/w in a homogeneous SFL or SFD powder.

We have estimated the enhanced reactivity due to surface accumulation of protein, using stability versus composition data for hGH:sucrose systems as a model^{185,186} and also using typical surface enrichment data for spray-dried proteins from the literature.¹⁷⁸ With hGH, as well as with other proteins,^{17,186–189} a plot of $\log(k_{\text{obs}})$ is essentially linear in weight fraction stabilizer. With the simulation, we use the log-linear rate constant-composition data for hGH:sucrose and evaluate the ratio of k_{obs} in a heterogeneous system to k_{obs} in a homogeneous system as a function of protein content. The calculations are made for hypothetical samples having various percentages of the total sample in the 50 Å deep surface region, or various percentages of “surface phase.” Note that the “5% surface phase” corresponds roughly to a SSA of 7 m²/g. Results of the simulation (Fig. 11) suggest that:

- (a) Extensive chemical heterogeneity occurs in stabilizer-rich systems, with maximum

heterogeneity occurring at about 5% protein and 95% stabilizer.

- (b) Chemical heterogeneity may lead to inferior stability in stabilizer-rich systems. The enhancement in reactivity (or loss of stability) relative to a homogeneous system may be more than an order of magnitude for high specific surface area materials.

Of course, this calculation represents only the chemical heterogeneity effect. If the protein undergoes additional unfolding because of the air–water or ice–water interface, this destabilizing effect would add to the effect evaluated by the simulation above.

For samples prepared by SFD and lyophilization, Webb et al.¹⁶ compared the SSA and surface composition of formulations of rhIFN- γ with trehalose (5:95, respectively) in the presence and absence of Tween 20. Additionally, aggregation in the formulations was measured initially and after

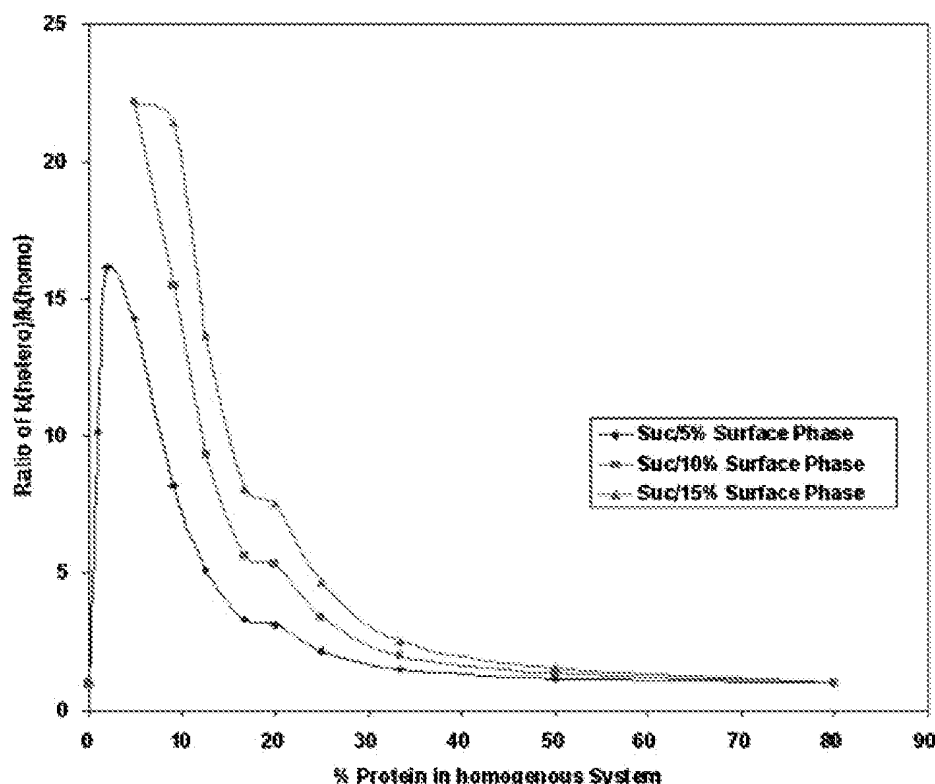


Figure 11. Ratio of homogeneous to heterogeneous degradation rates. Model is sucrose and hGH at 40°C (physical aggregation data), and data from Reference 177 for surface enrichment of protein in freeze-dried material. All ratios go to unity at both extremes of protein concentration and appear to be a maximum at around 5% protein. Calculations with aggregation of hGH with trehalose give qualitatively the same result but with less enhancement.

Table 2. Chemical Heterogeneity for a Formulation of rhIFN- γ (5 mg/mL) with Trehalose (9.5%)

Drying Method	0.12% Tween	Aggregation Rate Constant (k_{obs})* (% Aggregate/Week)	SSA (m^2/g) \pm SD**	Protein Surface Coverage (% w/w)	Total Protein Surface Accumulation (% $P_{total-surface}$)
Lyophilization	No	0.01	0.68 \pm 0.06	10.9	0.8
	Yes	0.00	0.70 \pm 0.06	3.2	0.2
Spray lyophilization	No	0.07	12.3 \pm 0.8	39	53
	Yes	0.04	13.9 \pm 1.1	8.3	13

Buffer was 10 mM potassium phosphate (pH 7.5) with or without Tween 20 (T20). Solutions were freeze dried and spray lyophilized. Data from Webb et al.¹⁶ Based on formulation composition, rhIFN- γ surface coverage would be \sim 5% w/w in a homogeneous SFL or SFD powder.

*Estimate for rate constant is based on two time points after storage at 45°C for 2 weeks.

**SD, Standard deviation.

2 weeks storage at 45°C. Table 2 summarizes their data, in addition to estimates of both the $\%P_{total-surface}$ and aggregation rate constant (k_{obs}) for each formulation. Lyophilized preparations were much more stable than SFD preparations. Additionally, SSA was roughly 20 times higher in SFD formulations versus lyophilized formulations. The degradation rate constant, k_{obs} , appeared to correlate well with $\%P_{total-surface}$. k_{obs} was highest for the surfactant-free SFD preparation, consistent with the highest SSA and highest protein surface coverage, and therefore highest $\%P_{total-surface}$ value. On the other hand, k_{obs} was lowest for the lyophilized preparation with surfactant, consistent with the lowest SSA, lowest protein surface coverage, and therefore lowest $\%P_{total-surface}$ value.

In summary, a combination of factors may cause composition heterogeneity in dried formulations. Evidence from studies on different proteins strongly suggests that composition heterogeneity varies depending on drying method, stabilizer used, and use of surfactant. However, the implications of this composition heterogeneity on different solid-state properties of the product, including long-term storage stability, are still inadequately documented.

CONCLUSIONS

Selection of the appropriate formulation and drying method are crucial factors for stability of amorphous pharmaceuticals. By understanding variations in thermal history of a pharmaceutical glass due to the drying method, and/or by measuring molecular mobility, one can qualitatively determine the impact of drying method on both in-process and storage stability (physical and/or chemical stability) of the active ingredient. Surface composition, specific surface area, and separation of components during drying of protein formulations are other critical factors to consider, particularly when dealing with preparations with a very high SSA.

REFERENCES

1. Garcia-Villaluenga JP, Seoane B, Diaz-Calleja R. 1997. Thermomechanical and diffusive studies in films prepared from copolymers of ethylene-1-octene subject to longitudinal and transversal induced stretching. *Polymer* 38:3827–3836.

2. Kitabatake N, Indo K, Doi E. 1989. Changes in interfacial properties of hen egg ovalbumin caused by freeze-drying and spray-drying. *J Agric Food Chem* 37:905–910.
3. Diaz-Maroto MC, Perez-Coello MS, Cabezudo MD. 2002. Effect of different drying methods on the volatile components of parsley (*Petroselinum crispum* L.). *Eur Food Res Technol* 215:227–230.
4. Monsoor MA. 2005. Effect of drying methods on the functional properties of soy hull pectin. *Carbohydr Polym* 61:362–367.
5. Shitanda D, Wanjala NV. 2006. Effect of different drying methods on the quality of jute (*Corchorus olitorius* L.). *Dry Technol* 24:95–98.
6. Jacquot M, De Donato P, Poncelet D. 2002. Physicochemical characterisation of the lactoperoxidase system powders: Comparison of two drying techniques. *Powder Technol* 128:205–212.
7. Marques L, Silveira A, Freire J. 2006. Freire, freeze-drying characteristics of tropical fruits. *Dry Technol* 24:457–463.
8. Marreto RN, Freire JT, Freitas LAP. 2006. Drying of pharmaceuticals: The applicability of spouted beds. *Dry Technol* 24:327–338.
9. Schroeder AL, Schwarz HW. 1949. Low-temperature vacuum dehydration. *Chem Eng Prog* 45:370–376.
10. Franks F, Hatley RHM, Mathias SF. 1991. Materials science and the production of shelf-stable biologicals. *BioPharm* (Duluth MN United States) 4:38, 40–42, 55.
11. Mattern M, Winter G, Lee G. 1997. Formulation of proteins in vacuum-dried glasses. Part 1. Improved vacuum-drying of sugars using crystallizing amino acids. *Eur J Pharm Biopharm* 44:177–185.
12. Mattern M, Winter G, Lee G. 1999. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. *Pharm Dev Technol* 4:199–208.
13. Truong-Le V. Preservation of bioactive materials by freeze dried foam. 2003. USA: Medimmune Vaccines, Inc., Application: WO. p 72.
14. Bronshtein V. Preservation by foam formation. 1998. USA: Universal Preservation Technologies, Inc., Application: WO. p 13.
15. Bronshtein V. Preservation of biopharmaceuticals and vaccines by vaporization. 2005. USA: Application: WO. p 53.
16. Webb SD, Golledge SL, Randolph TW. 2002. Surface adsorption of recombinant human interferon- γ in lyophilized and spray-lyophilized formulations. *J Pharm Sci* 91:1474–1487.
17. Sane SU, Wong R, Hsu CC. 2004. Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: Correlating structural changes with long-term stability. *J Pharm Sci* 93:1005–1018.
18. Allison SD, Randolph TW, Carpenter JF. 1998. Effects of drying methods and additives on structure and function of actin: Mechanisms of dehydration-induced damage and its inhibition. *Arch Biochem Biophys* 358:171–181.
19. Maa Y-F, Prestrelski SJ. 2000. Biopharmaceutical powders: Particle formation and formulation considerations. *Curr Pharm Biotechnol* 1:283–302.
20. Maa Y-F, Nguyen P-A, Hsu CC. 1999. Protein inhalation powders: Spray drying vs spray freeze drying. *Pharm Res* 16:249–254.
21. Killeen M. 1988. Spray drying and spray congealing of pharmaceuticals. In: Swarbrick J, Boylan JC, editors. *Encyclopedia of pharmaceutical technology*, Vol. 14. NY, NY: Marcel Dekker. pp 207–221.
22. Filkova I, Cedik P. 1984. Nozzle atomization in spray drying. *Adv Dry* 3:181–215.
23. Okamoto H, Todo H, Danjo K. 2002. Dry powders for pulmonary delivery of peptides and proteins. *Kona* 20:71–83.
24. Lee G. 2002. Spray-drying of proteins. *Pharm Biotechnol* 13 (Rational Design of Stable Protein Formulations):135–158.
25. Broadhead J, Rouan SKE, Rhodes CT. 1992. The spray drying of pharmaceuticals. *Drug Dev Ind Pharm* 18:1169–1206.
26. Chen T. 1992. Formulation concerns of protein drugs. *Drug Dev Ind Pharm* 18:1311–1354.
27. Elversson J, Millqvist-Fureby A. 2005. Particle size and density in spray drying-effects of carbohydrate properties. *J Pharm Sci* 94:2049–2060.
28. Maa YF, Hsu CC. 1997. Feasibility of protein spray coating using a fluid-bed Wurster processor. *Biotechnol Bioeng* 53:560–566.
29. Masters K. 1990. Understanding and applying spray dryers in chemical processing. *Powder Bulk Eng* 4:36–38, 40, 42, 44.
30. Maa Y-F, Ameri M, Chen D. 2004. Spray-coating for biopharmaceutical powder formulations: Beyond the conventional scale and its application. *Pharm Res* 21:515–523.
31. Maa Y-F, Nguyen P-A, Hsu CC. 1996. Spray-coating of rhDNase on lactose: Effect of system design, operational parameters and protein formulation. *Int J Pharm* 144:47–59.
32. DePaz RA, Dale DA, Randolph TW. 2002. Effects of drying methods and additives on the structure, function, and storage stability of subtilisin: Role of protein conformation and molecular mobility. *Enzyme Microb Technol* 31:765–774.
33. Maa Y-F, Costantino HR, Hsu CC. 1997. The effect of operating and formulation variables on the morphology of spray-dried protein particles. *Pharm Dev Technol* 2:213–223.

34. Worrall EE, Litamoi JK, Ayelet G. 2000. Xerovac: An ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. *Vaccine* 19: 834–839.
35. Kramer M, Sennhenn B, Lee G. 2002. Freeze-drying using vacuum-induced surface freezing. *J Pharm Sci* 91:433–443.
36. Pikal MJ. 1995. Lyophilization. In: Swarbrick J, Boylan JC, editors. *Encyclopedia of pharmaceutical technology*. NY: Marcel Dekker. pp 275–303.
37. Pikal MJ. 1994. Freeze-drying of proteins: Process, formulation, and stability. *ACS Symposium Series* 567 (Formulation and Delivery of Proteins and Peptides):120–133.
38. Pikal MJ. 1990. Freeze-drying of proteins. Part I: Process design. *BioPharm* (Duluth MN United States) 3:18–20, 22–24, 26–28.
39. Nail SL, Jiang S, Knopp SA. 2002. Fundamentals of freeze-drying. *Pharm Biotechnol* 14 (Development and Manufacture of Protein Pharmaceuticals):281–360.
40. Carpenter JF, Pikal MJ, Randolph TW. 1997. Rational design of stable lyophilized protein formulations: Some practical advice. *Pharm Res* 14: 969–975.
41. Carpenter JF, Manning MC, editors. 2002. Rational design of stable protein formulations: Theory and practice. [In: *Pharm Biotechnol*, 2002; 13]. p 203.
42. Costantio HR, Pikal MJ, editors. 2004. Lyophilization of biopharmaceuticals. [In: *Biotechnol: Pharm Aspects*, 2004; 2]. p 686.
43. Seager H. 1998. Drug-delivery products and the Zydis fast-dissolving dosage form. *J Pharm Pharmacol* 50:375–382.
44. Randolph TW. 1997. Phase separation of excipients during lyophilization: Effects on protein stability. *J Pharma Sci* 86:1198–1203.
45. Costantino HR, Pikal MJ, editors. 2004. Lyophilization of biopharmaceuticals. [In: *Biotechnol Pharm Aspects*, 2004; 2]. p 686.
46. Pikal MJ, Shah S, Lang JE. 1983. Physical chemistry of freeze-drying: Measurement of sublimation rates for frozen aqueous solutions by a microbalance technique. *J Pharm Sci* 72:635–650.
47. Lu X, Pikal MJ. 2004. Freeze-drying of mannitol-trehalose-sodium chloride-based formulations: The impact of annealing on dry layer resistance to mass transfer and cake structure. *Pharm Dev Technol* 9:85–95.
48. Akers MJ, Milton N, Nail SL. 1995. Glycine crystallization during freezing: The effects of salt form, pH, and ionic strength. *Pharm Res* 12:1457–1461.
49. Maa Y-F, Ameri M, Chen D. 2004. Influenza vaccine powder formulation development: Spray-freeze-drying and stability evaluation. *J Pharm Sci* 93:1912–1923.
50. Jovanovic N, Bouchard A, Jiskoot W. 2004. Stabilization of proteins in dry powder formulations using supercritical fluid technology. *Pharm Res* 21:1955–1969.
51. Forbes RT, Sloan R, York P. 1998. Production of stable protein particles: A comparison of freeze, spray and supercritical drying. *World Congress on Particle Technology* 3. Brighton, UK: July 6–9, 1998. pp 1857–1869.
52. Sellers SP, Clark GS, Sievers RE, Carpenter JF. 2001. Dry powders of stable protein formulations from aqueous solutions prepared using supercritical CO₂-assisted aerosolization. *J Pharm Sci* 90:785–797.
53. Zaks A. 1992. Protein-water interactions. Role in protein structure and stability. *Pharm Biotechnol* 2 (Stability of Protein Pharmaceuticals, Pt. A): 249–271.
54. Pikal MJ, Dellerman K, Roy ML. 1992. Formulation and stability of freeze-dried proteins: Effects of moisture and oxygen on the stability of freeze-dried formulations of human growth hormone. *Dev Biol Stand* 74 (Biol. Prod. Freeze-Drying Formulation):21–38.
55. Hageman MJ. 1988. The role of moisture in protein stability. *Drug Dev Ind Pharm* 14:2047–2070.
56. Tang X, Pikal MJ. 2005. Measurement of the kinetics of protein unfolding in viscous systems and implications for protein stability in freeze-drying. *Pharm Res* 22:1176–1185.
57. Banga AK. 2006. Therapeutic peptides and proteins: Formulation, processing, and delivery systems, 2nd edition. Boca Raton, FL: CRC Press. p 324.
58. Mumenthaler M, Hsu CC, Pearlman R. 1994. Feasibility study on spray-drying protein pharmaceuticals: Recombinant human growth hormone and tissue-type plasminogen activator. *Pharm Res* 11:12–20.
59. Izutsu K, Yoshioka S, Takeda Y. 1990. Protein denaturation in dosage forms measured by differential scanning calorimetry. *Chem Pharm Bull* 38:800–803.
60. Labrude P, Rasolomanana M, Chaillot B. 1989. Protective effect of sucrose in spray drying of oxyhemoglobin. *J Pharm Sci* 78:223–229.
61. Stahl K, Claesson M, Lilliehorn P, Linden H, Backstrom K. 2002. The effect of process variables on the degradation and physical properties of spray dried insulin intended for inhalation. *Int J Pharm* 233:227–237.
62. Adler M, Lee G. 1999. Stability and surface activity of lactate dehydrogenase in spray-dried trehalose. *J Pharm Sci* 88:199–208.

63. Tang X, Pikal MJ. 2004. Design of freeze-drying processes for pharmaceuticals: Practical advice. *Pharm Res* 21:191–200.
64. Bam NB, Randolph TW, Cleland JL. 1995. Stability of protein formulations: Investigation of surfactant effects by a novel EPR spectroscopic technique. *Pharm Res* 12:2–11.
65. Shibata H. 2006. Vacuum drying of porous solids under supercooling. *Dry Technol* 24:541–550.
66. Chongprasert S, Griesser UJ, Nail SL. 1998. Effects of freeze-dry processing conditions on the crystallization of pentamidine isethionate. *J Pharm Sci* 87:1155–1160.
67. Milton N, Nail SL. 1996. The physical state of nafcillin sodium in frozen aqueous solutions and freeze-dried powders. *Pharm Dev Technol* 1:269–277.
68. Truong-Le V, Scherer T. 2005. High pressure spray-dry of bioactive materials. USA: Medimmune Vaccines, Inc., Application: WO. p 71.
69. Szkudlarek BA, Anchordoquy TJ, Rodriguez-Hornedo N. pH changes of phosphate buffer solutions during freezing and their influence on the stability of a model protein, lactate dehydrogenase. Book of Abstracts 211th ACS National Meeting, New Orleans, LA, March 24–28, 1996. p BIOT-138.
70. Heller MC, Carpenter JF, Randolph TW. 1999. Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation. *Biotechnol Bioeng* 63:166–174.
71. Strambini GB, Gabellieri E. 1996. Proteins in frozen solutions: Evidence of ice-induced partial unfolding. *Biophys J* 70:971–976.
72. Eckhardt BM, Oeswein JQ, Bewley TA. 1991. Effect of freezing on aggregation of human growth hormone. *Pharm Res* 8:1360–1364.
73. Hsu CC, Nguyen HM, Yeung DA, Brooks DA, Koe GS, Bewley TA, Pearlman R. 1995. Surface denaturation at solid-void interface—A possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. *Pharm Res* 12:69–77.
74. Chang BS, Kendrick BS, Carpenter JF. 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J Pharm Sci* 85:1325–1330.
75. Sarciaux J-M, Mansour S, Nail SL. 1999. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. *J Pharm Sci* 88:1354–1361.
76. Jiang S, Nail SL. 1998. Effect of process conditions on recovery of protein activity after freezing and freeze-drying. *Eur J Pharm Biopharm* 45:249–257.
77. Izutsu K, Yoshioka S, Terao T. 1993. Stability of b-galactosidase by amphiphilic additives during freeze-drying. *Int J Pharm* 90:187–194.
78. Abdul-Fattah A, Kalonia D, Pikal M. 2005. The effect of surfactant and protein levels on the specific surface area of freeze dried formulations. In American Association of Pharmaceutical Scientists Annual Meeting. Nashville, TN.
79. Hansson UB. 1969. Correlation between amount of aggregates formed on freezing of immunoglobulin G and protein concentration. *Acta Chemica Scandinavica* (1947–1973) 23:1828–1829.
80. Pearlman R, Nguyen TH. 1989. Formulation strategies for recombinant proteins: Human growth hormone and tissue plasminogen activator. In: Marshak D, Liu D, editors. *Therapeutic peptides and proteins: Formulations, delivery and targeting*, Current communications in molecular biology. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
81. Pikal MJ, Dellerman KM, Riggin RM. 1991. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm Res* 8:427–436.
82. Hatley RHM, Franks F, Day H. 1986. Subzero-temperature preservation of reactive fluids in the undercooled state. II. The effect on the oxidation of ascorbic acid of freeze concentration and undercooling. *Biophys Chem* 24:187–192.
83. Orii Y, Morita M. 1977. Measurement of the pH of frozen buffer solutions by using pH indicators. *J Biochem (Tokyo)* 81:163–168.
84. Shalaev EY, Johnson-Elton TD, Chang L, Pikal MJ. 2002. Thermophysical properties of pharmaceutically compatible buffers at sub-zero temperatures: Implications for freeze-drying. *Pharm Res* 19:195–201.
85. van den Berg L, Rose D. 1959. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: The reciprocal system $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4\text{-H}_2\text{O}$. *Arch Biochem Biophys* 81:319–329.
86. Hill JJ, Shalaev EY, Zografi G. 2005. Thermodynamic and dynamic factors involved in the stability of native protein structure in amorphous solids in relation to levels of hydration. *J Pharm Sci* 94:1636–1667.
87. Smeller L. 2002. Pressure-temperature phase diagrams of biomolecules. *Biochimica et Biophysica Acta Protein Struct Mol Enzymol* 1595:11–29.
88. Jonas J. 2002. High-resolution nuclear magnetic resonance studies of proteins. *Biochimica et Biophysica Acta Protein Struct Mol Enzymol* 1595:145–159.
89. Privalov PL. 1990. Cold denaturation of proteins. *Crit Rev Biochem Mol Biol* 25:281–305.
90. Franks F, Hatley RHM, Friedman HL. 1988. The thermodynamics of protein stability. *Cold*

- destabilization as a general phenomenon. *Biophys Chem* 31:307–315.
91. Greiff D. 1969. Comparative studies of the cryobiology of viruses classified according to their physicochemical characteristics. Freezing Drying Microorganisms, Pap. Conf. "Mech. Cell. Inj. Freezing Drying Microorganisms". pp 69–80.
 92. Rexroad J, Evans RK, Middaugh CR. 2005. Effect of pH and ionic strength on the physical stability of adenovirus type 5. *J Pharm Sci* 95:237–247.
 93. Rightsel WA, Greiff D. 1967. Freezing and freeze-drying of viruses. *Cryobiology* 3:423–431.
 94. Slade L, Levine H. 1991. Beyond water activity: Recent advances based on an alternative approach to the assessment of food quality and safety. *Crit Rev Food Sci Nutr* 30:115–360.
 95. Searles JA, Carpenter JF, Randolph TW. 2001. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine T_g' in pharmaceutical lyophilization. *J Pharm Sci* 90:872–887.
 96. Randolph TW, Searles JA. 2002. Freezing and annealing phenomena in lyophilization: Effects upon primary drying rate, morphology, and heterogeneity. *Am Pharm Rev* 5:40, 42, 44–46.
 97. Heller MC, Carpenter JF, Randolph TW. 1997. Manipulation of lyophilization-induced phase separation: Implications for pharmaceutical proteins. *Biotechnol Prog* 13:590–596.
 98. Surface activity of proteins: Chemical and physicochemical modifications. 1996. Magdassi S, editor. New York, NY: Elsevier. 327p.
 99. Tripp BC, Magda JJ, Andrade JD. 1995. Adsorption of globular proteins at the air/water interface as measured via dynamic surface tension: Concentration dependence, mass-transfer considerations, and adsorption kinetics. *J Colloid Interface Sci* 173:16–27.
 100. Advances in food emulsions and foams. 1988. Dickinson E, Stainsby G, editors. London, UK: Elsevier. 397p.
 101. Dickinson E. 1999. Adsorbed protein layers at fluid interfaces: Interactions, structure and surface rheology. *Colloids Surf B Biointerfaces* 15:161–176.
 102. Tzannis ST, Prestrelski SJ. 1999. Activity-stability considerations of trypsinogen during spray drying: Effects of sucrose. *J Pharm Sci* 88:351–359.
 103. Katakam M, Bell LN, Banga AK. 1995. Effect of surfactants on the physical stability of recombinant human growth hormone. *J Pharm Sci* 84: 713–716.
 104. Oeswein JQ, Shire SJ. Physical biochemistry of protein drugs. *Adv Parenter Sci* 1990. 4 (Pept. Protein Drug Delivery): 167–202.
 105. Norde W, Giacomelli CE. 2000. BSA structural changes during homomolecular exchange between the adsorbed and the dissolved states. *J Biotechnol* 79:259–268.
 106. Abdul-Fattah A, Pikal M, Lechuga-Ballesteros D. 2002. Unpublished observations.
 107. Chou DK, Randolph TW, Manning MC. 2005. Effects of tween 20 and tween 80 on the stability of albutropin during agitation. *J Pharm Sci* 94:1368–1381.
 108. Maa Y-F, Nguyen P-AT, Hsu SW. 1998. Spray-drying of air-liquid interface-sensitive recombinant human growth hormone. *J Pharm Sci* 87: 152–159.
 109. Adler M, Unger M, Lee G. 2000. Surface composition of spray-dried particles of bovine serum albumin/trehalose/surfactant. *Pharm Res* 17: 863–870.
 110. Randolph Theodore W, Jones LaToya S. 2002. Surfactant-protein interactions. *Pharm Biotechnol* 13:159–175.
 111. Ananthapadmanabhan KP. 1993. Protein-surfactant interactions. *Interact Surfactants Polym Proteins* 319–365.
 112. Rashin AA, Honig B. 1984. On the environment of ionizable groups in globular proteins. *J Mol Biol* 173:515–521.
 113. Pikal MJ. 2004. Mechanisms of protein stabilization during freeze-drying and storage: The relative importance of thermodynamic stabilization and glassy state relaxation dynamics. *Drugs Pharm Sci* 137 (Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products):63–107.
 114. Carpenter JF, Izutsu K-I, Randolph TW. 2004. Freezing- and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives. *Drugs Pharm Sci* 137 (Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products):147–186.
 115. Izutsu K, Yoshioka S, Terao T. 1994. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. *Chem Pharm Bull* 42:5–8.
 116. Carpenter JF, Prestrelski SJ, Arakawa T. 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. *Arch Biochem Biophys* 303: 456–464.
 117. Lechuga-Ballesteros D, Abdul-Fattah A, Bennett DB. 2003. Properties and stability of a liquid crystal form of cyclosporine—The first reported naturally occurring peptide that exists as a thermotropic liquid crystal. *J Pharm Sci* 92: 1821–1831.
 118. Yu L. 2001. Amorphous pharmaceutical solids: Preparation, characterization and stabilization. *Adv Drug Deliv Rev* 48:27–42.
 119. Lu Q, Zografi G. 1998. Phase behavior of binary and ternary amorphous mixtures containing indomethacin, citric acid, and PVP. *Pharm Res* 15: 1202–1206.

120. Dong A, Meyer JD, Carpenter JF. 2000. Comparative Fourier transform infrared and circular dichroism spectroscopic analysis of α 1-proteinase inhibitor and ovalbumin in aqueous solution. *Arch Biochem Biophys* 383:148–155.
121. Costantino HR, Schwendeman SP, Langer R. 1996. The secondary structure and aggregation of lyophilized tetanus toxoid. *J Pharm Sci* 85:1290–1293.
122. Costantino HR, Griebenow K, Klibanov AM. 1995. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. *Biochimica et Biophysica Acta Protein Struct Mol Enzymol* 1253:69–74.
123. Prestrelski SJ, Tedeschi N, Carpenter JF. 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys J* 65:661–671.
124. Kendrick BS, Dong A, Carpenter JF. 1996. Quantitation of the area of overlap between second-derivative amide I infrared spectra to determine the structural similarity of a protein in different states. *J Pharm Sci* 85:155–158.
125. Li X, Nail SL. 2005. Kinetics of glycine crystallization during freezing of sucrose/glycine excipient systems. *J Pharm Sci* 94:625–631.
126. Izutsu K, Yoshioka S, Carpenter JF. 1996. Effects of sugars and polymers on crystallization of poly(ethylene glycol) in frozen solutions: Phase separation between incompatible polymers. *Pharm Res* 13:1393–1400.
127. Heller MC, Carpenter JF, Randolph TW. 1996. Effects of phase separating systems on lyophilized hemoglobin. *J Pharm Sci* 85:1358–1362.
128. Carpenter JF, Prestrelski SJ, Dong A. 1998. Application of infrared spectroscopy to development of stable lyophilized protein formulations. *Eur J Pharm Biopharm* 45:231–238.
129. Prestrelski SJ, Pikal KA, Arakawa T. 1995. Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy. *Pharm Res* 12:1250–1259.
130. Chang BS, Beauvais RM, Carpenter JF. 1996. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: Glass transition and protein conformation. *Arch Biochem Biophys* 331:249–258.
131. Angell CA. 1995. Formation of glasses from liquids and biopolymers. *Science (Washington, DC)* 267:1924–1935.
132. Pikal MJ. 2002. Chemistry in solid amorphous matrices: Implication for biostabilization. In *Amorphous food and pharmaceutical systems*. Cambridge, UK: The Royal Society of Chemistry. pp 257–272.
133. Hancock BC, Zografi G. 1997. Characteristics and significance of the amorphous state in pharmaceutical systems. *J Pharm Sci* 86:1–12.
134. Hancock BC, Shamblin SL, Zografi G. 1995. Molecular mobility of amorphous pharmaceutical solids below their glass transition temperatures. *Pharm Res* 12:799–806.
135. Shamblin SL, Hancock BC, Pikal MJ. 2000. Interpretation of relaxation time constants for amorphous pharmaceutical systems. *J Pharm Sci* 89:417–427.
136. Pauly S, Kammer HW. 1994. Physical aging in copolymer based blends. *Polym Netw Blends* 4:93–99.
137. Hill SA, MacNaughtan W, Whitcombe MJ. 2005. The effect of thermal history on the maillard reaction in a glassy matrix. *J Agric Food Chem* 53:10213–10218.
138. Tong P, Zografi G. 1999. Solid-state characteristics of amorphous sodium indomethacin relative to its free acid. *Pharm Res* 16:1186–1192.
139. Roy ML, Pikal MJ, Maloney AM. 1992. The effects of formulation and moisture on the stability of a freeze-dried monoclonal antibody-vinca conjugate: A test of the WLF glass transition theory. *Dev Biol Stand* 74 (Biol. Prod. Freeze-Drying Formulation): 323–340.
140. Guo Y, Byrn SR, Zografi G. 2000. Physical characteristics and chemical degradation of amorphous quinapril hydrochloride. *J Pharm Sci* 89:128–143.
141. Cicerone MT, Tellington A, Sokolov A. 2003. Substantially improved stability of biological agents in dried form: The role of glassy dynamics in preservation of biopharmaceuticals. *BioProc Int* 1:36–38, 40, 42, 44, 46–47.
142. Pikal MJ, Rigsbee DR. 1997. The stability of insulin in crystalline and amorphous solids: Observation of greater stability for the amorphous form. *Pharm Res* 14:1379–1387.
143. Kawakami K, Pikal MJ. 2005. Calorimetric investigation of the structural relaxation of amorphous materials: Evaluating validity of the methodologies. *J Pharm Sci* 94:948–965.
144. Streefland L, Auffret AD, Franks F. 1998. Bond cleavage reactions in solid aqueous carbohydrate solutions. *Pharm Res* 15:843–849.
145. Yoshioka S, Aso Y, Kojima S. 2001. Usefulness of the Kohlrausch-Williams-Watts stretched exponential function to describe protein aggregation in lyophilized formulations and the temperature dependence near the glass transition temperature. *Pharm Res* 18:256–260.
146. Duddu SP, Dal Monte PR. 1997. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. *Pharm Res* 14:591–595.

147. Parker R, Gunning YM, Ring SG. 2002. Glassy state dynamics, its significance for biostabilisation and the role of carbohydrates. *Special Publication Royal Society of Chemistry 281 (Amorphous Food and Pharmaceutical Systems)*:73–87.
148. Liu J, Rigsbee DR, Pikal MJ. 2002. Dynamics of pharmaceutical amorphous solids: The study of enthalpy relaxation by isothermal microcalorimetry. *J Pharm Sci* 91:1853–1862.
149. Yoshida H, Kanbara H, Kobayashi Y. 1983. Effect of hydrogen bond formation on physical aging of ethylene-vinyl alcohol copolymers with annealing below T_g. *Sen'i Gakkaishi* 39:T512–T518.
150. Baysal C, Atilgan AR. 2005. Relaxation kinetics and the glassiness of native proteins: Coupling of timescales. *Biophys J* 88:1570–1576.
151. Yoshioka S, Aso Y, Kojima S. 2004. Temperature- and glass transition temperature-dependence of bimolecular reaction rates in lyophilized formulations described by the Adam-Gibbs-Vogel equation. *J Pharm Sci* 93:1062–1069.
152. Duddu SP, Zhang G, Dal Monte PR. 1997. The relationship between protein aggregation and molecular mobility below the glass transition temperature of lyophilized formulations containing a monoclonal antibody. *Pharm Res* 14:596–600.
153. Yoshioka S, Aso Y, Kojima S. 1996. Determination of molecular mobility of lyophilized bovine serum albumin and g-globulin by solid-state ¹H NMR and relation to aggregation-susceptibility. *Pharm Res* 13:926–930.
154. Hodge IM. 1995. Physical aging in polymer glasses. *Science (Washington, DC)* 267:1945–1947.
155. Struik LCE. 1978. Physical aging in amorphous polymers and other materials. New York, NY: Elsevier. 242p.
156. Lammert AM, Lammert RM, Schmidt SJ. 1999. Physical aging of maltose glasses as measured by standard and modulated differential scanning calorimetry. *J Thermal Anal Calorimetry* 55: 949–975.
157. Moynihan CT. 1995. Structural relaxation and the glass transition. *Rev Mineralogy* 32:1–19.
158. Shalaev E, Zografi G. 2002. The concept of 'structure' in amorphous solids from the perspective of the pharmaceutical sciences. *Special Publication Royal Society of Chemistry 281 (Amorphous Food and Pharmaceutical Systems)*:11–30.
159. Deschenes LA, Vanden Bout DA. 2001. Single-molecule studies of heterogeneous dynamics in polymer melts near the glass transition. *Science* 292:255–258.
160. Madsen LD, De Wilton AC, Mercier JS. 1991. Examination of the stability of borophosphosilicate glass films. *Chemtronics* 5:35–42.
161. Mardaleishvili IR, Anisimov VM. 1987. b-Transition and relaxation of impurities in molecules in their reactions in glass polymers. *Izvestiya Akademii Nauk SSSR Seriya Khimicheskaya* 1431–1432.
162. Levine H, editor. 2002. Amorphous Food and Pharmaceutical Systems. Proceedings of the Symposium held at Churchill College, Cambridge 15–17 May 2001. [In: *Spec Publ R Soc Chem*, 2002; 281]. p 351.
163. Cicerone MT, Soles CL. 2004. Fast dynamics and stabilization of proteins: Binary glasses of trehalose and glycerol. *Biophys J* 86:3836–3845.
164. Cicerone MT, Pikal MJ, Chang L. 2005. Fast dynamics as a diagnostic for excipients in preservation of dried proteins. *Am Pharm Rev* 8:22, 24–27.
165. Yoshioka S, Aso Y, Kojima S. 2004. Temperature- and glass transition temperature-dependence of bimolecular reaction rates in lyophilized formulations described by the Adam-Gibbs-Vogel equation. *J Pharm Sci* 93:1062–1069.
166. Yoshioka S, Miyazaki T, Aso Y. 2006. b-Relaxation of insulin molecule in lyophilized formulations containing trehalose or dextran as a determinant of chemical reactivity. *Pharm Res* 23:961–966.
167. Yoshioka S, Aso Y. 2005. A quantitative assessment of the significance of molecular mobility as a determinant for the stability of lyophilized insulin formulations. *Pharm Res* 22:1358–1364.
168. Yoshioka S, Aso Y, Miyazaki T. 2006. Negligible contribution of molecular mobility to the degradation rate of insulin lyophilized with poly(vinylpyrrolidone). *J Pharm Sci* 95:939–943.
169. Buera MP, Rossi S, Chirife J. 1999. DSC confirmation that vitrification is not necessary for stabilization of the restriction enzyme EcoRI dried with saccharides. *Biotechnol Prog* 15:577–579.
170. Davidson P, Sun WQ. 2001. Effect of sucrose/raffinose mass ratios on the stability of co-lyophilized protein during storage above the T_g. *Pharm Res* 18:474–479.
171. Meyer A, Dimeo RM, Neumann DA. 2003. The high-flux backscattering spectrometer at the NIST Center for Neutron Research. *Rev Sci Instruments* 74:2759–2777.
172. Yoshioka S. 2004. Molecular mobility of freeze-dried formulations as determined by NMR relaxation, and its effect on storage stability. *Drugs Pharm Sci* 137 (Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products):187–212.
173. Millqvist-Fureby A, Malmsten M, Bergenstahl B. 1999. Surface characterization of freeze-dried protein/carbohydrate mixtures. *Int J Pharm* 191: 103–114.
174. Millqvist-Fureby A, Burns N, Bergenstahl B. 1999. Surface activity at the air-water interface in relation to surface composition of spray-dried milk protein-stabilized emulsions. *Special Publication*

- Royal Society of Chemistry 227 (Food Emulsions and Foams):236–245.
175. Millqvist-Fureby A, Malmsten M, Bergenstahl B. 1999. Surface characterisation of freeze-dried protein/carbohydrate mixtures. *Int J Pharm* 191: 103–114.
 176. Millqvist-Fureby A, Malmsten M, Bergenstahl B. 1999. Spray-drying of trypsin- surface characterisation and activity preservation. *Int J Pharm* 188: 243–253.
 177. Yu Z, Johnston KP, Williams RO. 2006. Spray freezing into liquid versus spray-freeze drying: Influence of atomization on protein aggregation and biological activity. *Eur J Pharm Sci* 27:9–18.
 178. Faeldt P, Bergenstahl B. 1994. The surface composition of spray-dried protein-lactose powders. *Colloids Surf A Physicochem Eng Aspects* 90:183–190.
 179. Faeldt P, Bergenstahl B, Carlsson G. 1993. The surface coverage of fat on food powders analyzed by ESCA (electron spectroscopy for chemical analysis). *Food Struct* 12:225–234.
 180. Briggs D, Grant JT. 2003. Surface analysis by Auger and X-ray photoelectron spectroscopy. Chichester, UK: IM Publications. 899p.
 181. Abdul-Fattah A, Pikal M, Lechuga-Ballesteros D. 2005. Composition heterogeneity effects due to variation in processing and formulation of Methionyl-human Growth Hormone. In AAPS National Biotechnology Conference. San Francisco, CA.
 182. Elversson J, Millqvist-Fureby A, Elofsson U. 2003. Droplet and particle size relationship and shell thickness of inhalable lactose particles during spray drying. *J Pharm Sci* 92:900–910.
 183. Landstrom K, Alsins J, Bergenstahl B. 2000. Competitive protein adsorption between bovine serum albumin and b-lactoglobulin during spray-drying. *Food Hydrocolloids* 14:75–82.
 184. Abdul-Fattah A, Kalonia D, Pikal M, Lechuga-Ballesteros D. 2005. The surface composition of spray dried and freeze dried protein formulations with varying molecular weight stabilizers. In American Association of Pharmaceutical Scientists Annual Meeting. Nashville, TN.
 185. Pikal M, Rigsbee D, Roy M. Unpublished observations.
 186. Chang L, Shepherd D, Pikal MJ. 2005. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J Pharm Sci* 94:1427–1444.
 187. Breen ED, Curley JG, Shire SJ. 2001. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm Res* 18:1345–1353.
 188. Cleland JL, Lam X, Carpenter JF. 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *J Pharm Sci* 90:310–321.
 189. Maury M, Murphy K, Lee G. 2005. Spray-drying of proteins: Effects of sorbitol and trehalose on aggregation and FT-IR amide I spectrum of an immunoglobulin G. *Eur J Pharm Biopharm* 59: 251–261.